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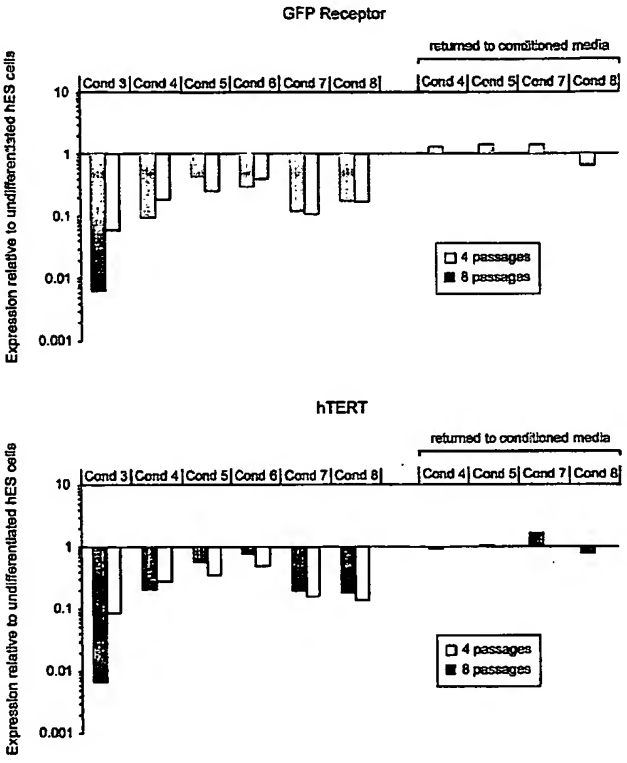

(57) Abstract: This disclosure provides a system for qualifying embryonic stem cells intended for human therapy. A large-scale sequencing project has identified important markers that are characteristic of undifferentiated pluripotent cells. Combinations of these markers can be used to validate the self-renewing capacity of ES cells, and their ability to differentiate into tissue types suitable for regenerative medicine. The marker system of this invention has been used to screen feeder cells, media additives, and culture conditions that promote proliferation of stem cells without differentiation. A culture system optimized by following these markers is suitable for rapid expansion of undifferentiated cells from existing lines, or the derivation of new lines that are equally apposite for clinical use.

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A MARKER SYSTEM FOR CHARACTERIZING UNDIFFERENTIATED HUMAN EMBRYONIC STEM CELLS

BACKGROUND

A promising development in the field of regenerative medicine has been the isolation and propagation of human stem cells from the early embryo. These cells have two very special properties: First, unlike other normal mammalian cell types, they can be propagated in culture almost indefinitely, providing a virtually unlimited supply. Second, they can be used to generate a variety of tissue types of interest as a source of replacement cells and tissues for use in therapy.

Thomson et al. (Science 282:114, 1998; U.S. Patent 6,200,806) were the first to successfully isolate and propagate embryonic stem cells from human blastocysts. Gearhart and coworkers derived human embryonic germ cell lines from fetal gonadal tissue (Shamblott et al., Proc. Natl. Acad. Sci. USA 95:13726, 1998;U.S. Patent 6,090,622).

International Patent Publication WO 99/20741 (Geron Corp.) describes methods and materials for the growth of primate-derived primordial stem cells. International Patent Publication WO 01/51616 (Geron Corp.) provides techniques for growth and differentiation of human pluripotent stem cells. An article by Xu et al. (Nature Biotechnology 19:971, 2001) describes feeder-free growth of undifferentiated human embryonic stem cells. Lebkowski et al. (Cancer J. 7 Suppl. 2:S83, 2001) discuss the culture, differentiation, and genetic modification of human embryonic stem cell for regenerative medicine applications. These publications report exemplary culture methods for propagating human embryonic stem cells in an undifferentiated state, and their use in preparing cells for human therapy.

Markers for identifying undifferentiated pluripotent stem cells include SSEA-4, Tra-1-60, and Tra-1-81 (Thomson et al. and Gearhart et al., supra). They also express human telomerase reverse transcriptase, and the POU transcription factor Oct 3/4 (WO 01/51616; Amit et al., Dev. Biol. 227:271, 2000; Xu et al., supra).

Loring et al. (Restor. Neurol. Neurosci. 18:81, 2001) review gene expression profiles of embryonic stem cells and ES-derived neurons. Pesce et al. (Bioessays 20:722, 1998) comment on the potential role of transcription factor Oct-4 in the totipotent germ-line cycle of mice. Gajovic et al. (Exp. Cell Res. 242:138, 1998) report that genes expressed after retinoic acid-mediated differentiation of embryoid bodies are likely to be expressed during embryo development. Zur Nieden et al. (Toxicol. in Vitro 15:455, 2001) propose certain molecular markers for embryonic stem cells. Henderson et al. (Stem Cells 20:329, 2002) report that pre-implantation human embryos and ES cells have comparable expression of SSEAs. Tanaka et al. (Genome Res. 12:1921, 2002) profile gene expression in mouse ES cells to identify candidate genes associated with pluripotency and lineage specificity. Draper et al. (J. Anat. 299:249, 2002) review change of surface antigens of human embryonic stem cells upon differentiation in culture.

Kelly et al. (Mol Reprod. Dev. 56:113, 2000) report DNA microarray analyses of genes regulated during the differentiation of embryonic stem cells. Woltjen et al. (Nucl. Acids Res. 28:E41, 2000) report retro-recombination screening of a mouse embryonic stem cell genomic library. Monk et al. (Oncogene

20:8085, 2001) list human embryonic genes re-expressed in cancer cells. Tanaka et al. (Genome Res. 12:1921, 2002) discuss gene expression profiling of embryo-derived stem cells, and candidate genes putatively associated with pluripotency and lineage specificity. Monk et al. report developmental genes identified by differential display (Reprod. Fertil. Dev. 13:51, 2001). Natale et al. (Reprod. 122:687, 2001) characterize bovine blastocyst gene expression patterns by differential display RT-PCR.

Fan et al. (Dev. Biol. 210:481, 1999) propose that forced expression of the homeobox-containing gene *Pem* blocks differentiation of embryonic stem cells. Abdel-Rahman et al. (Hum. Reprod. 10:2787, 1995) report the effect of expressing transcription regulating genes in human preimplantation embryos. Jackson et al. (J. Biol. Chem. 277:38683, 2002) describe the cloning and characterization of *Ehox*, a homeobox gene that reportedly plays a role in ES cell differentiation.

The following disclosure provides new markers and marker combinations that are effective means to identify, characterize, qualify, and control differentiation of pluripotent cells.

SUMMARY OF THE INVENTION

This invention identifies a number of genes that are up- or down-regulated during the course of differentiation of early-stage pluripotent stem cells obtained from primates, exemplified by human embryonic stem cells. As a consequence, the genes are differentially expressed in undifferentiated versus differentiated cells. This property confers special benefit on these genes for identification, characterization, culturing, differentiation, and manipulation of stem cells and their progeny, and other cells that express the same markers.

One aspect of this invention is a system for assessing a culture of undifferentiated primate pluripotent stem (pPS) cells or their progeny, in which expression of one or more of the identified markers listed in the disclosure is detected or measured. The level of expression can be measured in isolation or compared with any suitable standard, such as undifferentiated pPS cells maintained under specified conditions, progeny at a certain stage of differentiation, or stable end-stage differentiated cells, such as may be obtained from the ATCC. Depending on whether the marker(s) are up- or down-regulated during differentiation, presence of the markers is correlated with the presence or proportion of undifferentiated or differentiated cells in the population.

An exemplary (non-limiting) combination suitable for qualifying cultures of pPS cells are markers of the undifferentiated phenotype selected from Cripto, gastrin-releasing peptide (GRP) receptor, podocalyxin-like protein, hTERT and/Oct 3/4 (POU domain, class 5 transcription factor), in various combinations. Other cell markers can be measured in conjunction, including any of the newly described differentiation markers listed in this disclosure, or traditional pPS cell markers like SSEA-4 and Tra-1-60. In addition, markers of various differentiated cell phenotypes can be assayed as a measure of contaminating cells. Early stage cell types include stromal cells (marked by CD44 and Vimentin), fibroblasts, mesenchymal cells, and embryoid body cells. The markers can be detected or quantified at the mRNA level by PCR amplification, at the protein or enzyme product level by antibody assay, or by any suitable technique.

The marker system of this invention can be used for quantifying the proportion of undifferentiated pPS cells or differentiated cells in the culture; for assessing the ability of a culture system or component

thereof (such as a soluble factor, culture medium, or feeder cell) to maintain pPS cells in an undifferentiated state; for assessing the ability of a culture system or component thereof to cause differentiation of pPS cells into a culture of lineage-restricted precursor cells or terminally differentiated cells; or for any other worthwhile purpose. This invention includes kits and the use of specific reagents in order to measure the expression of the markers whenever appropriate.

This invention also provides a system assessing the growth characteristics of a cell population by detecting or measuring expression of one or more of the differentially expressed marker genes identified in this disclosure. This can be applied not only to various types of pPS cells and progenitor cells in various stages of differentiation, but also to clinical samples from a disease condition associated with abnormal cell growth. Renewed expression of markers of a relatively undifferentiated phenotype may be diagnostic of disease conditions such as cancer, and can serve as a means by which to target therapeutic agents to the disease site.

The marker system can also be used to regulate gene expression. Transcriptional control elements for the markers will cause an operatively linked encoding region to be expressed preferentially in undifferentiated or differentiated cells. For example, the encoding sequence can be a reporter gene (such as a gene that causes the cells to emit fluorescence), a positive selection marker (such as a drug resistance gene), or a negative selection marker. Vector constructs comprising recombinant elements linked in this fashion can be used to positively select or deplete undifferentiated, differentiated, or cancerous cells from a mixed population or in vivo, depending on the nature of the effector gene and whether transcription is up- or down-regulated during differentiation. They can also be used to monitor culture conditions of pPS cells, differentiation conditions, or for drug screening.

The marker system of this invention can also be used to sort differentiated cells from less differentiated cells. The marker can be used directly for cell separation by adsorption using an antibody or lectin, or by fluorescence activated cell sorting. Alternatively, these separation techniques can be effected using a transcription promoter from the marker gene in a promoter-reporter construct.

The marker system of this invention can be used to map differentiation pathways or influence differentiation. Markers suited for this purpose may act as transcription regulators, or encode products that enhance cell interaction in some fashion. pPS cells or their differentiated progeny are genetically altered to increase expression of one or more of the identified genes using a transgene, or to decrease expression, for example, using an antisense or siRNA construct. Alternatively, gene products involved in cell interaction or signaling can be added directly to the culture medium. The effect of this can be to help maintain the transfected cell in the undifferentiated state, promote differentiation in general, or direct differentiation down a particular pathway.

Another aspect of the invention are methods for identifying these and other genes that are up- or down-regulated upon differentiation of any cell type. The methods involve comparing expression libraries obtained from the cells before and after differentiation, by sequencing transcripts in each of the libraries, and identifying genes that have statistically significant differences in the relative number of transcripts (as a percentage of transcripts in each library) at a confidence level of 67%, 95%, or 98%. The method can be enhanced by creating assemblies in which different sequences are counted for the same transcript if they are known to correspond to a single transcript according to previously compiled data.

Amongst the differentially expressed markers identified in this disclosure are 39 nucleotide sequences which are not present in their entirety in the UniGene database. These are listed in this

disclosure as SEQ. ID NOs:101 to 139. This invention includes novel nucleic acids consisting of or containing any of these sequences or the complementary sequences, and novel fragments thereof. This invention also includes novel polypeptides encoded in these sequences (made either by expressing the nucleic acid or by peptide synthesis), antibodies specific for the polypeptides (made by conventional techniques or through a commercial service), and use of these nucleic acids, peptides, and antibodies for any industrial application.

Also embodied in this invention are culture conditions and other cell manipulations identified using the marker system of this invention that are suitable for maintaining or proliferating pPS cells without allowing differentiation, or causing them to differentiate in a certain fashion. Culture conditions tested and validated according to this invention are illustrated in the example section.

Other embodiments of the invention will be apparent from the description that follows.

DRAWINGS

Figure 1 shows the profile of genes preferentially expressed in undifferentiated pluripotent stem cells, upon preliminary differentiation of the cells by culturing in retinoic acid or DMSO. Level of gene expression at the mRNA level was measured by real-time PCR assay. Any of the genes showing substantial down-regulation upon differentiation can be used to characterize the undifferentiated cell population, and culture methods suitable for maintaining them in an undifferentiated state.

Figure 2 shows the level of expression of five genes in hES cells, compared with fully differentiated cells. This five-marker panel provides robust qualification of the undifferentiated phenotype.

Figure 3 show results of an experiment in which hES cells of the H1 line were maintained for multiple passages in different media. Medium conditioned with feeder cells provides factors effective to allow hES cells to proliferate in culture without differentiating. However, culturing in unconditioned medium leads to decreased percentage of cells expressing CD9, and the classic hES cell marker SSEA-4.

Figure 4 illustrates the sensitivity of hTERT, Oct 3/4, Cripto, GRP receptor, and podocalyxin-like protein (measured by real-time PCR) as a means of determining the degree of differentiation of the cells. After multiple passages in unconditioned medium, all five markers show expression that has been downregulated by 10 to 10^4-fold.

Figure 5 shows results of an experiment in which the hES cell line H1 was grown on different feeder cell lines: mEF = mouse embryonic fibroblasts; hMSC = human mesenchymal stem cells; UtSMC = uterine smooth muscle cells; WI-38 = human lung fibroblasts. As monitored using Cripto, the hMSC is suitable for use as feeder cells to promote hES cell proliferation without differentiation.

Figure 6 shows results of an experiment in which different media were tested for their ability to promote growth of hES cells without proliferation. The test media were not preconditioned, but supplemented with 8-40 ng/mL bFGF, with or without stem cell factor, Flt3 ligand, or LIF. Effective combinations of factors (Conditions 4 to 8) were identified by following the undifferentiated phenotype using the markers of this invention. Alterations in expression profiles were temporary and reversible, showing that the cells are still undifferentiated.

Figure 7 shows analysis of the undifferentiated hES cell markers SSEA-4, TRA 1-60 and Oct-4 by antibody staining and flow cytometry. Oct-4 is detected by permeabilizing the cells before staining.

Figure 8 shows the results of the immunocytochemical analysis for stromal cell markers CD44, STRO-1 and Vimentin, which label cells in the hES cell culture that have undergone differentiation.

Figure 9 shows the relative gene expression levels for cell populations in which undifferentiated hES cells were mixed with BJ fibroblasts in increasing amounts.

DETAILED DESCRIPTION

The propensity of pluripotent stem cells to differentiate spontaneously has made it challenging for investigators to work with these cells. Consistent cultures of undifferentiated stem cells are required to compare results obtained from multiple experiments performed within or between laboratories. Unfortunately, morphological characterization is subjective and especially difficult for cultures that often contain 10-20% differentiated cells. Nevertheless, having a set of standardized criteria will be important in qualifying these cells for use in clinical therapy.

The marker system identified in this disclosure provides the basis for establishing these standards. 148,453 different transcripts were amplified and sequenced from undifferentiated human embryonic stem cells, and three types of progeny. As a result of this sequencing effort, 532 genes were identified having substantially higher EST counts in undifferentiated cells, and 142 genes were identified having substantially higher EST counts after differentiation. Other differentially expressed genes were identified by microarray analysis of undifferentiated cells, compared with cells at the beginning of the differentiation process.

The system provided by this invention can be used not only to qualify populations of undifferentiated cells, but in other powerful ways of maintaining and manipulating cells described later in this disclosure. Culture systems have been identified and protocols have been developed to expand cultures of undifferentiated cells and produce commercially viable quantities of cells for use in research, drug screening, and regenerative medicine.

Definitions

"Pluripotent Stem cells" (pPS cells) are pluripotent cells that have the characteristic of being capable under appropriate conditions of producing progeny of several different cell types that are derivatives of all of the three germinal layers (endoderm, mesoderm, and ectoderm), according to a standard art-accepted test, such as the ability to form a teratoma in 8-12 week old SCID mice. The term includes both established lines of stem cells of various kinds, and cells obtained from primary tissue that are pluripotent in the manner described. For the purposes of this disclosure, the pPS cells are not embryonal carcinoma (EC) cells, and are not derived from a malignant source. It is desirable (but not always necessary) that the cells be euploid. Exemplary pPS cells are obtained from embryonic or fetal tissue at any time after fertilization.

"Human Embryonic Stem cells" (hES cells) are pluripotent stem cells derived from a human embryo in the blastocyst stage, or human pluripotent cells produced by artificial means (such as by

nuclear transfer) that have equivalent characteristics. Exemplary derivation procedures and features are provided in a later section.

hES cell cultures are described as "undifferentiated" when a substantial proportion (at least 20%, and possibly over 50% or 80%) of stem cells and their derivatives in the population display morphological characteristics of undifferentiated cells, distinguishing them from differentiated cells of embryo or adult origin. It is understood that colonies of undifferentiated cells within the population will often be surrounded by neighboring cells that are differentiated. It is also understood that the proportion of cells displaying the undifferentiated phenotype will fluctuate as the cells proliferate and are passaged from one culture to another. Cells are recognized as proliferating in an undifferentiated state when they go through at least 4 passages and/or 8 population doublings while retaining at least about 50%, or the same proportion of cells bearing characteristic markers or morphological characteristics of undifferentiated cells.

A "differentiated cell" is a cell that has progressed down a developmental pathway, and includes lineage-committed progenitor cells and terminally differentiated cells.

"Feeder cells" or "feeders" are terms used to describe cells of one type that are co-cultured with cells of another type, to provide an environment in which the cells of the second type can grow. hES cell populations are said to be "essentially free" of feeder cells if the cells have been grown through at least one round after splitting in which fresh feeder cells are not added to support the growth of pPS cells.

The term "embryoid bodies" refers to aggregates of differentiated and undifferentiated cells that appear when pPS cells overgrow in monolayer cultures, or are maintained in suspension cultures. Embryoid bodies are a mixture of different cell types, typically from several germ layers, distinguishable by morphological criteria and cell markers detectable by immunocytochemistry.

A cell "marker" is any phenotypic feature of a cell that can be used to characterize it or discriminate it from other cell types. A marker of this invention may be a protein (including secreted, cell surface, or internal proteins; either synthesized or taken up by the cell); a nucleic acid (such as an mRNA, or enzymatically active nucleic acid molecule) or a polysaccharide. Included are determinants of any such cell components that are detectable by antibody, lectin, probe or nucleic acid amplification reaction that are specific for the cell type of interest. The markers can also be identified by a biochemical or enzyme assay that depend on the function of the gene product. Associated with each marker is the gene that encodes the transcript, and the events that lead to marker expression.

A marker is said to be "preferentially expressed" in an undifferentiated or differentiated cell population, if it is expressed at a level that is at least 10 times higher (in terms of total gene product measured in an antibody or PCR assay) or 10 times more frequently (in terms of positive cells in the population). Markers that are expressed 100, 1,000, or 10,000 times higher or more frequently are increasingly more preferred.

The terms "polynucleotide" and "nucleic acid" refer to a polymeric form of nucleotides of any length. Included are genes and gene fragments, mRNA, cDNA, plasmids, viral and non-viral vectors and particles, nucleic acid probes, amplification primers, and their chemical equivalents. As used in this disclosure, the term polynucleotide refers interchangeably to double- and single-stranded molecules. Unless otherwise specified, any embodiment of the invention that is a polynucleotide encompasses both a double-stranded form, and each of the two complementary single-stranded forms known or predicted to make up the double-stranded form.

A cell is said to be "genetically altered" or "transfected" when a polynucleotide has been transferred into the cell by any suitable means of artificial manipulation, or where the cell is a progeny of the originally altered cell that has inherited the polynucleotide.

A "control element" or "control sequence" is a nucleotide sequence involved in an interaction of molecules that contributes to the functional regulation of a polynucleotide, including replication, duplication, transcription, splicing, translation, or degradation of the polynucleotide. "Operatively linked" refers to an operative relationship between genetic elements, in which the function of one element influences the function of another element. For example, an expressible encoding sequence may be operatively linked to a promoter that drives gene transcription.

The term "antibody" as used in this disclosure refers to both polyclonal and monoclonal antibody. The ambit of the term deliberately encompasses not only intact immunoglobulin molecules, but also such fragments and derivatives of immunoglobulin molecules that retain a desired binding specificity.

General Techniques

Methods in molecular genetics and genetic engineering are described generally in the current editions of Molecular Cloning: A Laboratory Manual, (Sambrook et al.); Oligonucleotide Synthesis (M.J. Gait, ed.); Animal Cell Culture (R.I. Freshney, ed.); Gene Transfer Vectors for Mammalian Cells (Miller & Calos, eds.); Current Protocols in Molecular Biology and Short Protocols in Molecular Biology, 3rd Edition (F.M. Ausubel et al., eds.); and Recombinant DNA Methodology (R. Wu ed., Academic Press). Antibody production is described in Basic Methods in Antibody Production and Characterization (Howard & Bethell eds., CRC Press, 2000).

A survey of relevant techniques is provided in such standard texts as DNA Sequencing (A.E. Barron , John Wiley, 2002), and DNA Microarrays and Gene Expression (P. Baldi et al., Cambridge U. Press, 2002). For a description of the molecular biology of cancer, the reader is referred to Principles of Molecular Oncology (M.H. Bronchud et al. eds., Humana Press, 2000); The Biological Basis of Cancer (R.G. McKinnel et al. eds., Cambridge University Press, 1998); and Molecular Genetics of Cancer (J.K. Cowell ed., Bios Scientific Publishers, 1999).

Sources of Stem Cells

This invention is based on observations made with established lines of hES cells. The markers are suitable for identifying, characterizing, and manipulating related types of undifferentiated pluripotent cells. They are also suitable for use with pluripotent cells obtained from primary embryonic tissue, without first establishing an undifferentiated cell line. It is contemplated that the markers described in this application will in general be useful for other types of pluripotent cells, including embryonic germ cells (U.S. Patents 6,090,622 and 6,251,671), and ES and EG cells from other mammalian species, such as non-human primates.

Embryonic Stem Cells

Embryonic stem cells can be isolated from blastocysts of members of primate species (U.S. Patent 5,843,780; Thomson et al., Proc. Natl. Acad. Sci. USA 92:7844, 1995). Human embryonic stem (hES) cells can be prepared from human blastocyst cells using the techniques described by Thomson et

al. (U.S. Patent 6,200,806; Science 282:1145, 1998; Curr. Top. Dev. Biol. 38:133 ff., 1998) and Reubinoff et al, Nature Biotech. 18:399, 2000. Equivalent cell types to hES cells include their pluripotent derivatives, such as primitive ectoderm-like (EPL) cells, outlined in WO 01/51610 (Bresagen).

hES cells can be obtained from human preimplantation embryos. Alternatively, in vitro fertilized (IVF) embryos can be used, or one-cell human embryos can be expanded to the blastocyst stage (Bongso et al., Hum Reprod 4: 706, 1989). Embryos are cultured to the blastocyst stage in G1.2 and G2.2 medium (Gardner et al., Fertil. Steril. 69:84, 1998). The zona pellucida is removed from developed blastocysts by brief exposure to pronase (Sigma). The inner cell masses are isolated by immunosurgery, in which blastocysts are exposed to a 1:50 dilution of rabbit anti-human spleen cell antiserum for 30 min, then washed for 5 min three times in DMEM, and exposed to a 1:5 dilution of Guinea pig complement (Gibco) for 3 min (Solter et al., Proc. Natl. Acad. Sci. USA 72:5099, 1975). After two further washes in DMEM, lysed trophectoderm cells are removed from the intact inner cell mass (ICM) by gentle pipetting, and the ICM plated on mEF feeder layers.

After 9 to 15 days, inner cell mass derived outgrowths are dissociated into clumps, either by exposure to calcium and magnesium-free phosphate-buffered saline (PBS) with 1 mM EDTA, by exposure to dispase or trypsin, or by mechanical dissociation with a micropipette; and then replated on mEF in fresh medium. Growing colonies having undifferentiated morphology are individually selected by micropipette, mechanically dissociated into clumps, and replated. ES-like morphology is characterized as compact colonies with apparently high nucleus to cytoplasm ratio and prominent nucleoli. Resulting ES cells are then routinely split every 1-2 weeks by brief trypsinization, exposure to Dulbecco's PBS (containing 2 mM EDTA), exposure to type IV collagenase (~200 U/mL; Gibco) or by selection of individual colonies by micropipette. Clump sizes of about 50 to 100 cells are optimal.

Propagation of pPS Cells in an Undifferentiated State

pPS cells can be propagated continuously in culture, using culture conditions that promote proliferation without promoting differentiation. Exemplary serum-containing ES medium is made with 80% DMEM (such as Knock-Out DMEM, Gibco), 20% of either defined fetal bovine serum (FBS, Hyclone) or serum replacement (US 20020076747 A1, Life Technologies Inc.), 1% non-essential amino acids, 1 mM L-glutamine, and 0.1 mM β-mercaptoethanol. Just before use, human bFGF is added to 4 ng/mL (WO 99/20741, Geron Corp.).

Traditionally, ES cells are cultured on a layer of feeder cells, typically fibroblasts derived from embryonic or fetal tissue. Embryos are harvested from a CF1 mouse at 13 days of pregnancy, transferred to 2 mL trypsin/EDTA, finely minced, and incubated 5 min at 37°C. 10% FBS is added, debris is allowed to settle, and the cells are propagated in 90% DMEM, 10% FBS, and 2 mM glutamine. To prepare a feeder cell layer, cells are irradiated to inhibit proliferation but permit synthesis of factors that support ES cells (~4000 rads γ-irradiation). Culture plates are coated with 0.5% gelatin overnight, plated with 375,000 irradiated mEFs per well, and used 5 h to 4 days after plating. The medium is replaced with fresh hES medium just before seeding pPS cells.

Scientists at Geron have discovered that pPS cells can be maintained in an undifferentiated state even without feeder cells. The environment for feeder-free cultures includes a suitable culture substrate, particularly an extracellular matrix such as Matrigel® or laminin. The pPS cells are plated at >15,000

cells cm^{-2} (optimally 90,000 cm^{-2} to 170,000 cm^{-2}). Typically, enzymatic digestion is halted before cells become completely dispersed (say, ~5 min with collagenase IV). Clumps of ~10 to 2,000 cells are then plated directly onto the substrate without further dispersal. Alternatively, the cells can be harvested without enzymes before the plate reaches confluence by incubating ~5 min in a solution of 0.5 mM EDTA in PBS. After washing from the culture vessel, the cells are plated into a new culture without further dispersal. In a further illustration, confluent human embryonic stem cells cultured in the absence of feeders are removed from the plates by incubating with a solution of 0.05% (wt/vol) trypsin (Gibco) and 0.053 mM EDTA for 5-15 min at 37ºC. The remaining cells in the plate are removed and the cells are triturated into a suspension comprising single cells and small clusters, and then plated at densities of 50,000-200,000 cells cm^{-2} to promote survival and limit differentiation.

Feeder-free cultures are supported by a nutrient medium containing factors that support proliferation of the cells without differentiation. Such factors may be introduced into the medium by culturing the medium with cells secreting such factors, such as irradiated (~4,000 rad) primary mouse embryonic fibroblasts, telomerized mouse fibroblasts, or fibroblast-like cells derived from pPS cells. Medium can be conditioned by plating the feeders at a density of ~5-6 $\times 10^4$ cm^{-2} in a serum free medium such as KO DMEM supplemented with 20% serum replacement and 4 ng/mL bFGF. Medium that has been conditioned for 1-2 days is supplemented with further bFGF, and used to support pPS cell culture for 1-2 days. Alternatively or in addition, other factors can be added that help support proliferation without differentiation, such as ligands for the FGF-2 or FGF-4 receptor, ligands for c-kit (such as stem cell factor), ligands for receptors associated with gp130, insulin, transferrin, lipids, cholesterol, nucleosides, pyruvate, and a reducing agent such as β-mercaptoethanol. Aspects of the feeder-free culture method are further discussed in International Patent Publications WO 99/20741, WO 01/51616; Xu et al., Nat. Biotechnol. 19:971, 2001; and PCT application PCT/US02/28200. Exemplary culture conditions tested and validated using the marker system of this invention are provided below in Example 5.

Under the microscope, ES cells appear with high nuclear/cytoplasmic ratios, prominent nucleoli, and compact colony formation with poorly discernable cell junctions. Conventional markers for hES cells are stage-specific embryonic antigen (SSEA) 3 and 4, and markers detectable using antibodies Tra-1-60 and Tra-1-81 (Thomson et al., Science 282:1145, 1998). Differentiation of pPS cells in vitro results in the loss of SSEA-4, Tra-1-60, and Tra-1-81 expression, and increased expression of SSEA-1.

Markers of undifferentiated pPS cells and their differentiated progeny

The tables and description provided later in this disclosure provide markers that distinguish undifferentiated pPS cells from their differentiated progeny.

Expression libraries were made from ES cells (WO 01/51616), embryoid bodies (WO 01/51616), and cells differentiated towards the hepatocyte (WO 01/81549) or neural cell (WO 01/88104) lineage. mRNA was reverse transcribed and amplified, producing expressed sequence tags (ESTs) occurring in frequency proportional to the level of expression in the cell type being analyzed. The ESTs were subjected to automatic sequencing, and counted according to the corresponding unique (non-redundant) transcript. A total of 148,453 non-redundant transcripts were represented in each of the 4 libraries. Genes were then identified as having a differential expression pattern if the number of EST counts of the transcript was statistically different between the libraries being compared.

In a parallel set of experiments, mRNA from each of the cell types was analyzed for binding to a broad-specificity EST-based microarray, performed according to the method described in WO 01/51616. . Genes were identified as having a differential expression pattern if they showed a comparatively different signal on the microarray.

Significant expression differences determined by EST sequencing, microarray analysis, or other observations were confirmed by real-time PCR analysis. The mRNA was amplified by PCR using specific forward and reverse primers designed from the GenBank sequence, and the amplification product was detected using labeled sequence-specific probes. The number of amplification cycles required to reach a threshold amount was then compared between different libraries.

Distinguishing markers fall into several categories. Those of particular interest include the following:

- Markers characteristically expressed at a higher level in undifferentiated pPS cells than any of the differentiated cells, indicating down-regulation during differentiation. The gene products may be involved in maintaining the undifferentiated phenotype.
- Markers characteristically expressed at a higher level in the three differentiated cell types than in the undifferentiated cells, indicating up-regulation during differentiation. The gene products may be involved in the general differentiation process.
- Markers characteristically expressed at a higher level in one of the differentiated cell types. The encoded genes may be involved in differentiation down restricted lineages.

Markers can also be classified according to the function of the gene product or its location in the cell. Where not already indicated, protein gene products can be predicted by referencing public information according to the GenBank accession number, or by translating the open reading frame after the translation start signal though the genetic code. Features of the markers listed can be determined by the descriptors give in the tables below, or by using the accession number or sequence data to reference public information. Marker groups of particular interest include the following:

- Secreted proteins — of interest, for example, because they can be detected by immunoassay of the culture supernatant, and may transmit signals to neighboring cells. Secreted proteins typically have an N-terminal signal peptides, and may have glycosylation sites.
- Surface membrane proteins — of interest, for example, because they can be used for cell-surface labeling and affinity separation, or because they act as receptors for signal transduction. They may have glycosylation sites and a membrane spanning region. A Markov model for predicting transmembrane protein topology is described by Krogh et al., J. Mol Biol. 305:567, 2001.
- Enzymes with relevant function. For example, enzymes involved in protein synthesis and cleavage or in apoptosis may influence differentiation. Glycosyltransferases decorate the cell membrane with distinguishing carbohydrate epitopes that may play a role in cellular adhesion or localization.
- Transcription regulatory factors — of interest for their potential to influence differentiation, as explained later in this disclosure. These factors sometimes have zinc fingers or other identifiable topological features involved in the binding or metabolism of nucleic acids.

Through the course of this work, the key signaling pathways Wnt, Sonic hedgehog (Shh), and Notch emerged as regulators of growth of pPS cells. Interestingly, these pathways have also been shown to play a role in the growth of tumor cells of various kinds, and in embryonic development of lower species.

Now that genes have been identified that are up-regulated or down-regulated upon differentiation, a number of commercial applications of these markers will be apparent to the skilled reader. The sections that follow provide non-limiting illustrations of how some of these embodiments can be implemented.

Use of cell markers to characterize pPS cells and their differentiated progeny

The markers provided in this disclosure can be used as a means to identify both undifferentiated and differentiated cells — either a population as a whole, or as individual cells within a population. This can be used to evaluate the expansion or maintenance of pre-existing cell populations, or to characterize the pluripotent nature (or lineage commitment) of newly obtained populations.

Expression of single markers in a test cell will provide evidence of undifferentiated or differentiated phenotype, according to the expression pattern listed later in this disclosure. A plurality of markers (such as any 2, 3, 4, 5, 6, 8, 10, 12, 15, or 20 markers from Tables 2-3 or 5-9) will provide a more detailed assessment of the characteristics of the cell. Expression of genes that are down-regulated and/or lack of expression of genes that are up-regulated upon differentiation correlates with a differentiated phenotype. Expression of genes that are up-regulated and/or lack of expression of genes that are down-regulated upon differentiation correlates with an undifferentiated phenotype. The markers newly identified in this disclosure may be analyzed together (with or without markers that were previously known) in any combination effective for characterizing the cell status or phenotype.

Exemplary combinations of markers are provided elsewhere in this disclosure. For determining the undifferentiated cell phenotype, combinations of markers like Cripto, gastrin-releasing peptide (GRP) receptor, podocalyxin-like protein (PODXL), and human telomerase reverse transcriptase (hTERT) are effective, either alone, or in combination with cell surface markers like SSEA-3, SSEA-4, Tra-1-60 and Tra-1-81, or intracellular markers like Oct 3/4. For determining differentiated cells, any marker can be used that is characteristic of contaminating cells that may be present. Depending on culture conditions, early stage non-specific hES cell differentiation generates cells having characteristics of stromal cells, fibroblasts, mesenchymal cells, embryoid body cells, and other cell types. Alternatively, a combination of markers characteristic of several types of cells can be used, as long as they are preferentially expressed in differentiated cells.

Tissue-specific markers can be detected using any suitable immunological technique — such as flow cytochemistry for cell-surface markers, or immunocytochemistry (for example, of fixed cells or tissue sections) for intracellular or cell-surface markers. Expression of a cell-surface antigen is defined as positive if a significantly detectable amount of antibody will bind to the antigen in a standard immunocytochemistry or flow cytometry assay, optionally after fixation of the cells, and optionally using a labeled secondary antibody or other conjugate to amplify labeling.

The expression of tissue-specific gene products can also be detected at the mRNA level by Northern blot analysis, dot-blot hybridization analysis, or by reverse transcriptase initiated polymerase chain reaction (RT-PCR) using sequence-specific primers in standard amplification methods. See U.S.

Patent No. 5,843,780 for further details. Sequence data for particular markers listed in this disclosure can be obtained from public databases such as GenBank.

These and other suitable assay systems are described in standard reference texts, such as the following: PCR Cloning Protocols, 2nd Ed (James & Chen eds., Humana Press, 2002); Rapid Cycle Real-Time PCR: Methods and Applications (C. Wittwer et al. eds., Springer-Verlag NY, 2002); Immunoassays: A Practical Approach (James Gosling ed., Oxford Univ Press, 2000); Cytometric Analysis of Cell Phenotype and Function (McCarthy et al. eds., Cambridge Univ Press, 2001). Reagents for conducting these assays, such as nucleotide probes or primers, or specific antibody, can be packaged in kit form, optionally with instructions for the use of the reagents in the characterization or monitoring of pPS cells, or their differentiated progeny.

Use of cell markers for clinical diagnosis

Stem cells regulate their own replenishment and serve as a source of cells that can differentiate into defined cell lineages. Cancer cells also have the ability to self-renew, but lack of regulation results in uncontrolled cellular proliferation. Three key signaling pathways, Wnt, Sonic hedgehog (Shh), and Notch, are known growth regulators of tumor cells. The genomics data provided in this disclosure indicate that all three of these pathways are active in hES cells.

It is a hypothesis of this invention that many of the markers discovered to be more highly expressed in undifferentiated pPS cells can also be up-regulated upon dedifferentiation of cells upon malignant transformation. Accordingly, this disclosure provides a system for evaluating clinical conditions associated with abnormal cell growth, such as hyperplasia or cancers of various kinds. Markers meeting the desired criteria include those contained in Tables 2, 5, 7 and 9.

Expression of each marker of interest is determined at the mRNA or protein level using a suitable assay system such as those described earlier; and then the expression is correlated with the clinical condition that the patient is suspected of having. As before, combinations of multiple markers may be more effective in doing the assessment. Presence of a particular marker may also provide a means by which a toxic agent or other therapeutic drug may be targeted to the disease site.

In a similar fashion, the markers of this invention can be used to evaluate a human or non-human subject who has been treated with a cell population or tissue generated by differentiating pPS cells. A histological sample taken at or near the site of administration, or a site to which the cells would be expected to migrate, could be harvested at a time subsequent to treatment, and then assayed to assess whether any of the administered cells had reverted to the undifferentiated phenotype. Reagents for conducting diagnostic tests, such as nucleotide probes or primers, or specific antibody, can be packaged in kit form, optionally with instructions for the use of the reagents in the determination of a disease condition.

Use of cell markers to assess and manipulate culture conditions

The markers and marker combinations of this invention provide a system for monitoring undifferentiated pPS cells and their differentiated progeny in culture. This system can be used as a quality control, to compare the characteristics of undifferentiated pPS cells between different passages or

different batches. It can also be used to assess a change in culture conditions, to determine the effect of the change on the undifferentiated cell phenotype.

Where the object is to produce undifferentiated cells, a decrease in the level of expression of an undifferentiated marker because of the alteration by 3-, 10-, 25-, 100- and 1000-fold is progressively less preferred. Corresponding increases in marker expression may be more beneficial. Moderate decreases in marker expression may be quite acceptable within certain boundaries, if the cells retain their ability to form progeny of all three germ layers is retained, and/or the level of the undifferentiated marker is relatively restored when culture conditions are returned to normal.

In this manner, the markers of this invention can be used to evaluate different feeder cells, extracellular matrixes, base media, additives to the media, culture vessels, or other features of the culture as illustrated in WO 99/20741 and PCT application PCT/US02/28200. Illustrations of this technique are provided below in Example 5 (Figures 3 to 6).

In a similar fashion, the markers of this invention can also be used to monitor and optimize conditions for differentiating cells. Improved differentiation procedures will lead to higher or more rapid expression of markers for the differentiated phenotype, and/or lower or more rapid decrease in expression of markers for the undifferentiated phenotype.

Use of cell markers to regulate gene expression

Differential expression of the markers listed in this disclosure indicates that each marker is controlled by a transcriptional regulatory element (such as a promoter) that is tissue specific, causing higher levels of expression in undifferentiated cells compared with differentiated cells, or vice versa. When the corresponding transcriptional regulatory element is combined with a heterologous encoding region to drive expression of the encoding region, then the expression pattern in different cell types will mimic that of the marker gene.

Minimum promoter sequences of many of the genes listed in this disclosure are known and further described elsewhere. Where a promoter has not been fully characterized, specific transcription can usually be driven by taking the 500 base pairs immediately upstream of the translation start signal for the marker in the corresponding genomic clone.

To express a heterologous encoding region according to this embodiment of the invention, a recombinant vector is constructed in which the specific promoter of interest is operatively linked to the encoding region in such a manner that it drives transcription of the encoding region upon transfection into a suitable host cell. Suitable vector systems for transient expression include those based on adenovirus and certain types of plasmids. Vectors for long-term expression include those based on plasmid lipofection or electroporation, episomal vectors, retrovirus, and lentivirus.

One application of tissue-specific promoters is expression of a reporter gene. Suitable reporters include fluorescence markers such as green fluorescent protein, luciferase, or enzymatic markers such as alkaline phosphatase and β-galactosidase. Other reporters such as a blood group glycosyltransferase (WO 02/074935), or Invitrogen's pDisplay™, create a cell surface epitope that can be counterstained with labeled specific antibody or lectin. pPS cells labeled with reporters can be used to follow the differentiation process directly, the presence or absence of the reporter correlating with the undifferentiated or differentiated phenotype, depending on the specificity of the promoter. This in turn can

be used to follow or optimize culture conditions for undifferentiated pPS cells, or differentiation protocols. Alternatively, cells containing promoter-reporter constructs can be used for drug screening, in which a test compound is combined with the cell, and expression or suppression of the promoter is correlated with an effect attributable to the compound.

Another application of tissue-specific promoters is expression of a positive or negative drug selection marker. Antibiotic resistance genes such as neomycin phosphotransferase, expressed under control of a tissue-specific promoter, can be used to positively select for undifferentiated or differentiated cells in a medium containing the corresponding drug (geneticin), by choosing a promoter with the appropriate specificity. Toxin genes, genes that mediate apoptosis, or genes that convert a prodrug into a toxic compound (such as thymidine kinase) can be used to negatively select against contaminating undifferentiated or differentiated cells in a population of the opposite phenotype (WO 02/42445; GB 2374076).

Promoters specific for the undifferentiated cell phenotype can also be used as a means for targeting cancer cells — using the promoter to drive expression of a gene that is toxic to the cell (WO 98/14593, WO 02/42468), or to drive a replication gene in a viral vector (WO 00/46355). For example, an adenoviral vector in which the GRPR promoter (AY032865) drives the E1a gene should specifically lyse cancer cells in the manner described in Majumdar et al., Gene Ther. 8:568, 2001. Multiple promoters for the undifferentiated phenotype can be linked for improved cancer specificity (USSN 10/206,447).

Other useful applications of tissue-specific promoters of this invention will come readily to the mind of the skilled reader.

Use of markers for cell separation or purification

Differentially expressed markers provided in this disclosure are also a means by which mixed cell populations can be separated into populations that are more homogeneous. This can be accomplished directly by selecting a marker of the undifferentiated or differentiated phenotype, which is itself expressed on the cell surface, or otherwise causes expression of a unique cell-surface epitope. The epitope is then used as a handle by which the marked cells can be physically separated from the unmarked cells. For example, marked cells can be aggregated or adsorbed to a solid support using an antibody or lectin that is specific for the epitope. Alternatively, the marker can be used to attach a fluorescently labeled antibody or lectin, and then the cell suspension can be subject to fluorescence-activated cell sorting.

An alternative approach is to take a tissue-specific promoter chosen based on its expression pattern (as described in the last section), and use it to drive transcription of a gene suitable for separating the cells. In this way, the marker from which the promoter is chosen need not itself be a cell surface protein. For example, the promoter can drive expression of a fluorescent gene, such as GFP, and then cells having the marked phenotype can be separated by FACS. In another example, the promoter drives expression of a heterologous gene that causes expression of a cell-surface epitope. The epitope is then used for adsorption-based separation, or to attach a fluorescent label, as already described.

Use of cell markers to influence differentiation

In another embodiment of this invention, the differentially expressed genes of this invention are caused to increase or decrease their expression level, in order to either inhibit or promote the differentiation process. Suitable genes are those that are believed in the normal case of ontogeny to be active in maintaining the undifferentiated state, active in the general process of differentiation, or active in differentiation into particular cell lineages. Markers of interest for this application are the following:

- Transcription factors and other elements that directly affect transcription of other genes, such as Forkhead box O1A (FOXO1A); Zic family member 3 (ZIC3); Hypothetical protein FLJ20582; Forkhead box H1 (FOXH1); Zinc finger protein, Hsal2; KRAB-zinc finger protein SZF1-1; Zinc finger protein of cerebellum ZIC2; and Coup transcription factor 2 (COUP-TF2). Other candidates include those marked in Tables 5 and 6 with the symbol "⊗", and other factors with zinc fingers or nucleic acid binding activity.
- Genes that influence cell interaction, such as those that encode adhesion molecules, and enzymes that make substrates for adhesion molecules
- Genes encoding soluble factors that transmit signals within or between cells, and specific receptors that recognize them and are involved in signal transduction.

One way of manipulating gene expression is to induce a transient or stable genetic alteration in the cells using a suitable vector, such as those already listed. Scientists at Geron Corp. have determined that the following constitutive promoters are effective in undifferentiated hES cells: for transient expression CMV, SV40, EF1α, UbC, and PGK; for stable expression, SV40, EF1α, UbC, MND and PGK. Expressing a gene associated with the undifferentiated phenotype may assist the cells to stay undifferentiated in the absence of some of the elements usually required in the culture environment. Expressing a gene associated with the differentiated phenotype may promote early differentiation, and/or initiate a cascade of events beneficial for obtaining a desired cell population. Maintaining or causing expression of a gene of either type early in the differentiation process may in some instances help guide differentiation down a particular pathway.

Another way of manipulating gene expression is to alter transcription from the endogenous gene. One means of accomplishing this is to introduce factors that specifically influence transcription through the endogenous promoter. Another means suitable for down-regulating expression at the protein level is to genetically alter the cells with a nucleic acid that removes the mRNA or otherwise inhibits translation (for example, a hybridizing antisense molecule, ribozyme, or small interfering RNA). Dominant-negative mutants of the target factor can reduce the functional effect of the gene product. Targeting a particular factor associated with the undifferentiated phenotype in this fashion can be used to promote differentiation. In some instances, this can lead to de-repression of genes associated with a particular cell type.

Where the gene product is a soluble protein or peptide that influences cell interaction or signal transduction (for example, cytokines like osteopontin and Cripto), then it may be possible to affect differentiation simply by adding the product to the cells — in either recombinant or synthetic form, or purified from natural sources. Products that maintain the undifferentiated phenotype can then be withdrawn from the culture medium to initiate differentiation; and products that promote differentiation can be withdrawn once the process is complete.

Since differentiation is a multi-step process, changing the level of gene product on a permanent basis may cause multiple effects. In some instances, it may be advantageous to affect gene expression in a temporary fashion at each sequential step in the pathway, in case the same factor plays different effects at different steps of differentiation. For example, function of transcription factors can be evaluated by changing expression of individual genes, or by invoking a high throughput analysis, using cDNAs obtained from a suitable library such as exemplified in Example 1. Cells that undergo an alteration of interest can be cloned and pulled from multi-well plates, and the responsible gene identified by PCR amplification.

The effect of up- or down-regulating expression of a particular gene can be determined by evaluating the cell for morphological characteristics, and the expression of other characteristic markers. Besides the markers listed later in this disclosure, the reader may want to follow the effect on particular cell types, using markers for later-stage or terminally differentiated cells. Tissue-specific markers suitable for this purpose are listed in WO 01/81549 (hepatocytes), WO 01/88104 (neural cells), PCT/US02/20998 (osteoblasts and mesenchymal cells), PCT/US02/22245 (cardiomyocytes), PCT/US02/39091 (hematopoietic cells), PCT/US02/39089 (islet cells), and PCT/US02/39090 (chondrocytes). Such markers can be analyzed by PCR amplification, fluorescence labeling, or immunocytochemistry, as already described. Promoter-reporter constructs based on the same markers can facilitate analysis when expression is being altered in a high throughput protocol.

The examples that follow are provided for further illustration, and are not meant to limit the claimed invention.

EXAMPLES

Example 1: An EST database of undifferentiated hES cells and their differentiated progeny

cDNA libraries were prepared from human embryonic stem (hES) cells cultured in undifferentiated form. cDNA libraries were also prepared from progeny, subject to non-specific differentiation as embryoid bodies (EBs), or taken through the preliminary stages of established differentiation protocols for neurons (preNEU) or hepatocytes (preHEP).

The hES cell lines H1, H7, and H9 were maintained under feeder-free conditions. Cultures were passaged every 5-days by incubation in 1 mg/mL collagenase IV for 5-10 min at 37°C, dissociated and seeded in clumps at 2.5 to 10×10^5 cells/well onto Matrigel™-coated six well plates in conditioned medium supplemented with 8 mg/mL bFGF. cDNA libraries were made after culturing for 5 days after the last passage.

EBs were prepared as follows. Confluent plates of undifferentiated hES cells were treated briefly with collagenase IV, and scraped to obtain small clusters of cells. Cell clusters were resuspended in 4 mL/well differentiation medium (KO DMEM containing 20% fetal bovine serum in place of 20% SR, and not preconditioned) on low adhesion 6-well plates (Costar). After 4 days in suspension, the contents of each well was transferred to individual wells pre-coated with gelatin. Each well was re-fed with 3 mL fresh differentiation medium every two days after replating. Cells were used for the preparation of cytoplasmic RNA on the eighth day after plating.

PreHEP cells were prepared based on the hepatocyte differentiation protocol described in WO 01/81549. Confluent wells of undifferentiated cells were prepared, and medium was changed to KO DMEM plus 20% SR + 1% DMSO. The medium was changed every 24 h, and cells were used for preparation of cytoplasmic RNA on day 5 of DMSO treatment.

PreNEU cells were prepared based on the neural differentiation protocol described in WO 01/88104. hES cells of the H7 line (p29) were used to generate EBs as described above except that 10 µM all-trans RA was included in the differentiation medium. After 4 days in suspension, EBs were transferred to culture plate precoated with poly-L-lysine and laminin. After plating, the medium was changed to EPFI medium. Cells were used for the preparation of cytoplasmic RNA after 3 days of growth in EPFI.

Partial 5' end sequences (an expressed sequence tag, or EST) were determined by conventional means for independent clones derived from each cDNA library. Overlapping ESTs were assembled into conjoined sequences.

TABLE 1: Non-redundant EST sequences

Library	Number of ESTs
hESC	37,081
EB	37,555
preHEP	35,611
preNEU	38,206
Total	148,453

All of the stem cell lines used for preparation of the expression libraries were originally isolated and initially propagated on mouse feeder cells. Accordingly, the libraries were analyzed to determine whether they were contaminated with murine retroviruses that had shed from the feeder cells and subsequently infected the stem cells. Three complete viral genomes were used in a BLAST search: Moloney murine leukemia virus, Friend murine leukemia virus, and murine type C retrovirus. No matches with a high score were found against any of the ESTs.

The sequences were then compared to the Unigene database of human genes. ESTs that were at least 98% identical, over a stretch of at least 150 nucleotides each, to a common reference sequence in Unigene, were assumed to be transcribed from the same gene, and placed into a common assembly. The complete set of 148,453 ESTs collapsed to a non-redundant set of 32,764 assemblies.

Example 2: Selection of marker genes specific for undifferentiated and differentiated cells

Candidate markers were selected from a database based on the imputed level of gene expression. The frequency of ESTs for any particular gene correlates with the abundance of that mRNA in the cells used to generate the cDNA library. Thus, a comparison of frequencies of ESTs among the libraries indicates the relative abundance of the associated mRNA in the different cell types.

Candidate molecular markers were selected from the expressed gene (EST) database from their greater abundance in undifferentiated hES cells, relative to differentiated hES cells. Genes were identified as having a differential expression pattern (being up- or down-regulated) during the differentiation process, if the count of ESTs sequenced in the undifferentiated cells was substantially different from the sum of ESTs in the three differentiated libraries.

Oct 3/4 (a POU domain-containing transcription factor) and telomerase reverse transcriptase (hTERT) are known to be expressed preferentially in undifferentiated hES cells (WO 01/51616). Other genes suitable for characterizing or manipulating the undifferentiated phenotype are those that are down-regulated upon differentiation with a significance of $p \leq 0.05$, as determined by the Fisher Exact Test (explained below). 193 genes were found to have 4-fold more ESTs in hES cells, relative to each of the three cell types. 532 genes were found that were 2-fold greater hES cells, with a confidence of over 95% as determined by the Fisher Exact Test, relative to the sum of ESTs of the three cell types (minimum of 4 ESTs in hES cells). The following markers are of particular interest:

TABLE 2: EST Frequency of Genes that are Down-regulated upon Differentiation of hES cells

Geron ID	GenBank ID	Name	EST counts			
			ES	EB	preHEP	preNEU
GA_10902	NM_024504	Pr domain containing 14 (PRDM14)	12	1	0	0
GA_11893	NM_032805	Hypothetical protein FLJ14549	25	0	0	0
GA_12318	NM_032447	Fibrillin3	6	0	0	0
GA_1322	NM_000142	Fibroblast growth factor receptor 3 precursor (FGFR-3)	9	1	5	1
GA_34679	NM_002015	Forkhead box o1a (FOXO1a)	4	0	1	1
GA_1470	NM_003740	potassium channel, subfamily K, member 5 (KCNK5), mRNA	4	0	0	1
GA_1674	NM_002701	Octamer-Binding Transcription Factor 3a (OCT-3A) (OCT-4)	24	1	2	0
GA_2024	NM_003212	Teratocarcinoma-derived growth factor 1 (CRIPTO)	20	1	0	0
GA_2149	NM_003413	Zic family member 3 (ZIC3)	7	0	1	0
GA_2334	NM_000216	Kallmann syndrome 1 sequence (KAL1)	5	0	1	0
GA_23552	NM_152742	hypothetical protein DKFZp547M109 (DKFZp547M109), mRNA	6	0	1	2
GA_2356	NM_002851	Protein tyrosine phosphatase, receptor-type, z polypeptide 1 (PTPRZ1),	10	0	0	0
GA_2357	NM_001670	Armadillo repeat protein deleted in velo-cardio-facial syndrome (ARVCF)	6	0	0	0
GA_23578	BM454360	AGENCOURT_6402318 NIH_MGC_85 Homo sapiens cDNA clone IMAGE:5497491 5', mRNA sequence	6	0	0	0

TABLE 2: EST Frequency of Genes that are Down-regulated upon Differentiation of hES cells

Geron ID	GenBank ID	Name	EST counts			
			ES	EB	preHEP	preNEU
GA_2367	NM_003923	Forkhead box H1 (FOXH1)	5	0	0	0
GA_2436	NM_004329	Bone morphogenetic protein receptor, type Ia (BMPR1A) (ALK-3)	7	3	1	1
GA_2442	NM_004335	Bone marrow stromal antigen 2 (BST-2)	13	0	2	3
GA_2945	NM_005232	Ephrin type-a receptor 1 (EPHA1)	5	1	1	1
GA_2962	NM_005314	Gastrin-releasing peptide receptor (GRP-R)	4	0	0	0
GA_2988	NM_005397	Podocalyxin-like (PODXL)	59	23	5	8
GA_3337	NM_006159	NELL2 (nel-like protein 2)	5	3	2	0
GA_3559	NM_005629	Solute carrier family 6, member 8 (SLC6A8)	5	1	0	1
GA_3898	NM_006892	DNA (cytosine-5-)-methyltransferase 3 beta (DNMT3B)	49	2	3	1
GA_5391	NM_002968	Sal-like 1 (SALL1),	7	1	1	0
GA_33680	NM_016089	Krab-zinc finger protein SZF1-1	15	0	1	0
GA_36977	NM_020927	KIAA1576 protein	9	2	1	0
GA_8723	NM_152333	Homo sapiens chromosome 14 open reading frame 69 (C14orf69), mRNA	14	1	1	3
GA_9167	AF308602	Notch 1 (N1)	6	2	1	0
GA_9183	NM_007129	Homo sapiens Zic family member 2 (odd-paired homolog, Drosophila) (ZIC2), mRNA	8	1	1	0
GA_35037	NM_004426	Homo sapiens polyhomeotic-like 1 (Drosophila) (PHC1), mRNA	34	9	5	4

Only one EST for hTERT was identified in undifferentiated hES cells and none were detected from the differentiated cells, which was not statistically significant. Thus, potentially useful markers that are expressed at low levels could have been omitted in this analysis, which required a minimum of four ESTs. It would be possible to identify such genes by using other techniques described elsewhere in this disclosure.

Three genes were observed from EST frequency queries that were of particular interest as potentially useful markers of hES cells. They were Teratocarcinoma-derived growth factor (Cripto), Podocalyxin-like (PODXL), and gastrin-releasing peptide receptor (GRPR). These genes were not only more abundant in undifferentiated cells, relative to differentiated hES cells, but also encoded for proteins expressed on the surface of cells. Surface markers have the added advantage that they could be easily detected with immunological reagents. ESTs for Cripto and GRPR were quite restricted to hES cells, with

one or zero ESTs, respectively, scored in any of the differentiated cells. PODXL ESTs were detected in all 4-cell types, but substantially fewer (2.5X -12X) in differentiated cells. All three markers retained a detectable level of expression in differentiated cultures of hES cells. There may be a low level of expression of these markers in differentiated cells, or the expression detected may be due to a small proportion of undifferentiated cells in the population. GABA(A) receptor, Lefty B, Osteopontin, Thy-1 co-transcribed, and Solute carrier 21 are other significant markers of the undifferentiated phenotype.

By similar reasoning, genes that show a higher frequency of ESTs in differentiated cells can be used as specific markers for differentiation. ESTs that are 2-fold more abundant in the sum of all three differentiated cell types (EBs, preHEP and preNEU cells) and with a p-value ≤ 0.05 as determined by the Fisher Exact Test, compared with undifferentiated hES cells are candidate markers for differentiation down multiple pathways. ESTs that are relatively abundant in only one of the differentiated cell types are candidate markers for tissue-specific differentiation. The following markers are of particular interest:

TABLE 3: EST Frequency of Genes that are Upregulated upon Differentiation

Geron ID	GenBank ID	Name	EST counts			
			ES	EB	preHEP	preNEU
GA_35463	NM_024298	Homo sapiens leukocyte receptor cluster (LRC) member 4 (LENG4), mRNA	0	4	9	8
GA_10492	NM_006903	Inorganic pyrophosphatase (PPASE)	0	5	5	6
GA_38563	NM_021005	Homo sapiens nuclear receptor subfamily 2, group F, member 2 (NR2F2), mRNA	0	9	8	9
GA_38570	NM_001844	Collagen, type II, alpha 1 (COL2A1), transcript variant 1		15	31	5
GA_1476	NM_002276	Keratin type I cytoskeletal 19 (cytokeratin 19)	1	26	14	38
GA_34776	NM_002273	Keratin type II cytoskeletal 8 (cytokeratin 8) (CK 8)	9	71	144	156
GA_1735	NM_002806	Homo sapiens proteasome (prosome, macropain) 26S subunit, ATPase, 6 (PSMC6), mRNA	1	7	7	8
GA_1843	NM_000982	60s ribosomal protein l21	1	7	48	42
GA_35369	NM_003374	Voltage-dependent anion-selective channel (VDAC-1)	1	5	6	10
GA_23117	NM_004772	P311 protein [Homo sapiens]	1	5	7	6
GA_2597	NM_138610	Homo sapiens H2A histone family, member Y (H2AFY), transcript variant 3, mRNA	1	5	5	14
GA_3283	NM_004484	Homo sapiens glypican 3 (GPC3), mRNA	1	6	7	12
GA_3530	NM_002539	Homo sapiens ornithine decarboxylase 1 (ODC1), mRNA	1	10	8	9
GA_4145	NM_002480	Protein phosphatase 1, regulatory(inhibitor) subunit 12A (PPP1R12A)	1	6	6	6

TABLE 3: EST Frequency of Genes that are Upregulated upon Differentiation

Geron ID	GenBank ID	Name	EST counts			
			ES	EB	preHEP	preNEU
GA_5992	NM_014899	Homo sapiens Rho-related BTB domain containing 3 (RHOBTB3), mRNA	0	10	7	13
GA_6136	NM_016368	Homo sapiens myo-inositol 1-phosphate synthase A1 (ISYNA1), mRNA	1	7	5	16
GA_6165	NM_015853	Orf (LOC51035)	1	5	9	5
GA_6219	NM_016139	16.7Kd protein (LOC51142),	1	5	13	14
GA_723	NM_005801	Homo sapiens putative translation initiation factor (SUI1), mRNA	1	14	15	19
GA_9196	NM_000404	Homo sapiens galactosidase, beta 1 (GLB1), transcript variant 179423, mRNA	0	6	10	7
GA_9649	NM_014604	Tax interaction protein 1 (TIP-1)	0	8	5	5

Example 3: Specificity of expression confirmed by real-time PCR

To verify the expression patterns of particular genes of interest at the mRNA level, extracts of undifferentiated hES cells and their differentiated progeny were assayed by real-time PCR. Cells were cultured for 1 week with 0.5% dimethyl sulfoxide (DMSO) or 500 nM retinoic acid (RA). The samples were amplified using sequence-specific primers, and the rate of amplification was correlated with the expression level of each gene in the cell population.

Taqman™ RT-PCR was performed under the following conditions: 1 x RT Master Mix (ABI), 300 nM for each primer, and 80 nM of probe, and 10 pg to 100 ng of total RNA in nuclease-free water. The reaction was conducted under default RT-PCR conditions of 48°C hold for 30 min, 95°C hold for 10 min, and 40 cycles of 95°C at 15 sec and 60°°C hold for 1 min. RNA was isolated by a guanidinium isothiocyanate method (RNAeasy™ kit, Qiagen) according to manufacturer's instructions, and subsequently DNAse treated (DNAfree™ kit, Ambion). Gene-specific primers and probes were designed by PrimerExpress™ software (Ver. 1.5, ABI). Probe oligonucleotides were synthesized with the fluorescent indicators 6-carboxyfluorescein (FAM) and 6-carboxy-tetramethylrhodamine (TAMRA) at the 5' and 3' ends, respectively. Relative quantitation of gene expression between multiple samples was achieved by normalization against endogenous18S ribosomal RNA (primer and probe from ABI) using the $\Delta\Delta C_T$ method of quantitation (ABI). Fold change in expression level was calculated as $2^{-\Delta\Delta CT}$.

The table below shows the results of this analysis. Since the cells have been cultured in RA and DMSO for a short period, they are at the early stages of differentiation, and the difference in expression level is less dramatic than it would be after further differentiation. Of particular interest for following or modulating the differentiation process are markers that show modified expression within the first week of differentiation by more than 2-fold (*), 5-fold (**), 10-fold (***), or 100-fold (****).

TABLE 4: Quantitative RT-PCR analysis of gene expression in hESC differentiation

	Geron ID	GenBank ID	Name	Fold Change	
				RA	DMSO
A.	GA_10902	NM_024504	Pr domain containing 14 (PRDM14) **	-1.9	-8.3
	GA_11893	NM_032805	Hypothetical protein FLJ14549 ***	-2.3	-10.0
	GA_12318	NM_032447	Fibrillin3		
	GA_1322	NM_000142	Fibroblast growth factor receptor 3 precursor (FGFR-3) *	1.5	2.3
	GA_1329	NM_002015	Forkhead box o1a (foxo1a) *	-1.6	-2.9
	GA_1470	NM_003740	Potassium channel subfamily k member 5 (TASK-2)	-1.6	1.0
	GA_1674	NM_002701	Octamer-binding transcription factor 3a (OCT-3A) (OCT-4) **	-3.7	-7.7
	GA_2024	NM_003212	Teratocarcinoma-derived growth factor 1 (CRIPTO) ***	-4.0	-12.5
	GA_2149	NM_003413	Zic family member 3 (ZIC3) **	-1.7	-5.3
	GA_2334	NM_000216	Kallmann syndrome 1 sequence (KAL1) *	-1.1	-2.5
	GA_23552	BC027972	Glypican-2 (cerebroglycan)	-1.5	-1.2
	GA_2356	NM_002851	Protein tyrosine phosphatase, receptor-type, z polypeptide 1 (PTPRZ1) *	-1.7	-3.3
	GA_2367	NM_003923	Forkhead box h1 (FOXH1) **	-1.8	-5.6
	GA_2436	NM_004329	Bone morphogenetic protein receptor, type Ia (BMPR1A) (ALK-3) *	-2.4	-2.4
	GA_2442	NM_004335	Bone marrow stromal antigen 2 (BST-2)	1.1	-1.9
	GA_2945	NM_005232	Ephrin type-a receptor 1 (EPHA1)	-1.3	-1.9
	GA_2962	NM_005314	Gastrin-releasing peptide receptor (GRP-R) **	-6.3	-9.1
	GA_2988	NM_005397	Podocalyxin-like (PODXL) *	-2.6	-4.3
	GA_3337	NM_006159	Nell2 (NEL-like protein 2)	-1.3	-1.3
	GA_3559	NM_005629	Solute carrier family 6, member 8 (SLC6A8)	-1.1	-1.1
	GA_420	X98834	Zinc finger protein, HSAL2 *	-1.4	-2.8
	GA_5391	NM_002968	Sal-like 1 (SALL1),	1.4	-1.3
	GA_6402	NM_016089	Krab-zinc finger protein SZF1-1 *	-1.8	-3.1
	GA_9167	AF308602	Notch 1 (N1)	1.3	1.0
	GA_9183	AF193855	Zinc finger protein of cerebellum ZIC2 *	1.0	-2.9
	GA_9443	NM_004426	Early development regulator 1 (polyhomeotic 1 homolog) (EDR1) **	-1.8	-5.6
B.	GA_9384	NM_020997	Left-right determination, factor b (LEFTB) **	-16.7	-25.0
	GA_12173	BC010641	Gamma-aminobutyric acid (GABA) A receptor,	-2.8	-5.6

TABLE 4: Quantitative RT-PCR analysis of gene expression in hESC differentiation

	Geron ID	GenBank ID	Name	Fold Change RA	Fold Change DMSO
			beta 3 **		
	GA_10513	NM_033209	Thy-1 co-transcribed ***	-12.5	-11.1
	GA_1631	NM_002941	Roundabout, axon guidance receptor, homolog 1 (ROBO1),	1.1	1.0
	GA_2753	NM_000582	Secreted phosphoprotein 1 (osteopontin) ***	-3.8	-10.0
	GA_32919	NM_133259	130 kDa leucine-rich protein (LRP 130)	-1.9	-1.9
	GA_28290	AK055829	FLJ31267 (acetylglucosaminyltransferase-like protein) *	-2.3	-4.5
C.	GA_28053	T24677	EST ****	<-100*	<-100*
	GA_26303	NM_138815	Hypothetical protein BC018070 ***	-3.2	-10.0
	GA_2028	NM_003219	Telomerase reverse transcriptase (TERT) *	-2.1	-2.3

Example 4: Selection of markers for monitoring ES cell differentiation

Genes that undergo up- or down-regulation in expression levels during differentiation are of interest for a variety of different commercial applications, as described earlier. This experiment provides an example in which certain genes were selected as a means to monitor the ability of culture conditions to maintain the undifferentiated cell phenotype — and hence, the pluripotent differentiation capability of the cells.

Particular genes were chosen from those identified as having differential expression patterns, because they are known or suspected of producing a protein gene product that is expressed at the cell surface, or is secreted. These attributes are helpful, because they allow the condition of the cells to be monitored easily either by antibody staining of the cell surface, or by immunoassay of the culture supernatant. Genes were chosen from the EST database (Groups 1), microarray analysis (Group 2), and other sources (Group 3).

TABLE 5: Additional Genes analyzed by real-time PCR

	Name	GenBank or ID No.
Group 1	Bone marrow stromal antigen	NM_004335
	Podocalyxin-like	NM_005397
	Rat GPC/ glypican-2 (cerebroglycan)	TA_5416486
	Potassium channel subfamily k member 5 (TASK-2)	NM_003740
	Notch 1 protein	AF308602

TABLE 5: Additional Genes analyzed by real-time PCR

	Name	GenBank or ID No.
	Teratocarcinoma-derived growth factor 1 (Cripto)	NM_003212
	Nel 1 like / NELL2 (Nel-like protein 2)	NM_006159
	Gastrin releasing peptide receptor	NM_005314
	Bone morphogenetic protein receptor	NM_004329
	ABCG2- ABC transporter	AY017168
	Solute carrier family 6, member 8 (SLC6A8)	NM_005629
	hTERT	NM_003219
	Oct 3/4 octamer-binding transcription factor 3a (oct-3a) (oct-4)	NM_002701
Group 2	Left-right determination factor b (LEFTB)	NM_020997
	Secreted phosphoprotein 1 (osteopontin)	NM_000582
	Gamma-aminobutyric acid (GABA) A receptor, beta 3	NM_021912
	Roundabout, axon guidance receptor, homologue 1 (ROBO1),	NM_002941
	Glucagon receptor	NM_00160
	Leucine-rich PPR-motif hum 130 kDa hum130leu 130kd Leu	M92439
	Thy-1 co-transcribed	NM_033209
	Solute carrier family 21	NM_016354
	LY6H lymphocyte antigen 6 complex locus H	NM_002347
	Plexin (PLXNB3)	NM_005393
	ICAM	NM_000201
Group 3	Rhodopsin	NM_000539
	Kallmann syndrome 1 sequence (KAL1)	NM_000216
	Armadillo repeat protein deleted in velo-cardio-facial syndrome (ARVCF)	NM_001670
	Ephrin type-a receptor 1 (EPHA1)	NM_005232

Figure 1 shows the decrease in expression of the genes in Group I (Upper Panel) and Group II (Lower Panel) in H9 hES cells after culturing for 7 days with RA or DM. Gene expression of rhodopsin and ICAM was below the limit of detection in differentiated cells. KAL1 and EPHA1 were not tested.

Besides hTERT and Oct 3/4, three other genes were selected as characteristic of the undifferentiated hES cell phenotype. They were Teratocarcinoma-derived growth factor (Cripto), Podocalyxin-like (PODXL), and gastrin-releasing peptide receptor (GRPR).

Figure 2 compares the level of expression of these five genes in hES cells with fully differentiated cells: BJ fibroblasts, BJ fibroblasts transfected to express hTERT (BJ-5TA), and 293

(human embryonic kidney) cells. The level of all markers shown was at least 10-fold higher, and potentially more than 10^2, 10^3, 10^4, 10^5, or 10^6-fold higher in pluripotent stem cells than fully differentiated cells. All five markers retained a detectable level of expression in differentiated cultures of hESC. It is not clear if there is lower level of expression of these markers in differentiated cells, or if the detectable expression derived from the undifferentiated cells in the population. The one exception observed in this experiment was the hTERT transgene, expressed at an elevated level as expected in the BJ-5TA cells.

High-level expression of Cripto, GRPR and PODXL in undifferentiated hES cells reveals interesting aspects of the biology of these cells. Cripto has been implicated in normal mammalian development and tumor growth. Cripto encodes a glycosylphosphoinositol anchored protein that contains an EGF repeat and a cysteine rich motif, which makes it a member of the EGF-CFC family. It has been demonstrated that Cripto serves as a co receptor for Nodal, which is essential for mesoderm and endoderm formation in vertebrate development (Yeo et al., Molecular Cell 7:949, 2001). The finding that Cripto is expressed preferentially on undifferentiated hESC suggests that Nodal is an important signaling molecule for stem cells, perhaps to promote survival and/or proliferation.

PODXL encodes for transmembrane sialoprotein that is physically linked to the cytoskeleton. PODXL is suspected to act as an inhibitor of cell-cell adhesion and has been implicated in the embryonic development of the kidney podocyte. The anti-adhesion properties of PODXL when expressed on undifferentiated hESC may be an important feature related to stem cell migration.

The receptor for gastrin releasing peptide (GRP) is a G-protein coupled receptor that mediates numerous biological effects of Bombesin-like peptides, including regulation of gut acid secretion and satiety. A critical role has also been established for GRP and GRPR in control growth of cultured cells and normal mammalian development. GRP and GRPR may be oncofetal antigens that act as morphogens in normal development and cancer.

Example 5: Use of cell markers to modify ES cell culture conditions

This example illustrates the utility of the differentially expressed genes identified according to this invention in the evaluation of culture environments suitable for maintaining pluripotent stem cells.

Figure 3 show results of an experiment in which hES cells of the H1 line were maintained for multiple passages in different media. Medium conditioned with feeder cells provides factors effective to allow hES cells to proliferate in culture without differentiating. However, culturing in unconditioned medium leads to loss of the undifferentiated phenotype, with an increasing percentage of the cells showing decreased expression of CD9 (a marker for endothelial cells, fibroblasts, and certain progenitor cells), and the classic hES cell marker SSEA-4.

Figure 4 illustrates the sensitivity of hTERT, Oct 3/4, Cripto, GRP receptor, and podocalyxin-like protein (measured by real-time PCR assay) as a means of determining the degree of differentiation of the cells. After 4 passages in unconditioned X-VIVO™ 10 medium containing 8 ng/mL bFGF, all 5 markers show expression that has been downregulated by about 10-fold. After 8 passages, expression has decreased by 10^2, 10^3, or 10^4-fold.

Figure 5 shows results of an experiment in which the hES cell line H1 was grown on different feeder cell lines: mEF = mouse embryonic fibroblasts; hMSC = human mesenchymal stem cells; UtSMC = human uterine smooth muscle cells; WI-38 = an established line of human lung fibroblasts. As

monitored by RT-PCR assay of Cripto, Oct 3/4, and hTERT, at least under the conditions used in this experiment, the hMSC are better substitutes for mEF feeders than the other cell lines tested.

Figure 6 shows results of an experiment in which different media were tested for their ability to promote growth of hES cells without differentiation. Expression of Podocalyxin-like protein, Cripto, GFP Receptor, and hTERT were measured by RT-PCR. The test media were not preconditioned, but supplemented with the growth factors as follows:

TABLE 6: Growth Conditions Tested for Marker Expression

Standard conditions:	DMEM preconditioned with mEF+ bFGF (8 ng/mL)
Condition 3	X-VIVO™ 10 + bFGF (8 ng/mL)
Condition 4	X-VIVO™ 10 + bFGF (40 ng/mL)
Condition 5	X-VIVO™ 10 + bFGF (40 ng/mL) + stem cell factor (SCF, 15 ng/mL)
Condition 6	X-VIVO™ 10 + bFGF (40 ng/mL) + Flt3 ligand (75 ng/mL)
Condition 7	X-VIVO™ 10 + bFGF (40 ng/mL) + LIF (100 ng/mL)
Condition 8	QBSF™-60 + bFGF (40ng/mL)

The results show that the markers selected to monitor the undifferentiated phenotype showed similar changes in each of these culture conditions. By all criteria, XVIVO 10™ supplemented according to Condition 6 was found to be suitable for culturing hES cells without having to be preconditioned. As shown on the right side, when cells were put back into standard conditioned medium after 8 passages in the test conditions, expression of all four markers returned essentially to original levels. This shows that alterations in expression profiles in media Conditions 4 to 8 are temporary and reversible — consistent with the cells retaining full pluripotency.

Example 6: Measuring undifferentiated cell markers by flow cytometry

Cells from the undifferentiated hES cell line H1 were grown in mEF conditioned medium in Matrigel® coated 6-well plates. Cells were harvested using 3.0 mL of 0.5 mM EDTA and resuspended in PBS containing 5% fetal calf serum and 0.05% NaN_3 at a concentration of 5×10^6 cells/mL. For SSEA-4 and TRA1-60 staining, 1 µg of antibody (Chemicon International) was used. Cells were incubated for a period of 30 min on ice followed by one wash with 2.0 mL of PBS-FCS buffer . Cell pellets were resuspended in 100 µl of fluorochrome conjugated secondary antibody. For intracellular Oct-4 staining, the cells were fixed with 2% PFA (final concentration) for 15 min at room temperature. After one wash, cells were resuspended in a permeabilization buffer (PBS-FCS plus 90% cold methanol) followed by 15 min in ice, washed again, and then resuspended the cell pellet in blocking solution (20% goat serum in permeabilization buffer). 0.5×10^6 or 1.0×10^6 permeabilized cells were stained with 1 µg of anti-Oct-4 antibody (Santa Cruz Biotechnology) in 10 µL of blocking solution, incubated on ice for 30 min. After rewashing, the cells were stained with labeled secondary antibody.

Figure 7 shows that SSEA-4, TRA 1-60 and Oct-4 markers were all strongly expressed on undifferentiated cells under these conditions. Solid areas in each panel indicate background staining observed with the respective isotype-matched controls. In fact, greater than 85% of hES cells expressed all three markers.

Example 7: Measuring differentiated cells using stromal markers

The extent of differentiation can be determined by detecting or measuring markers for undifferentiated cells, in combination with markers for differentiated cells of the type expected in early differentiation cultures — either by antibody staining, or by PCR amplification (Taqman™), or by a combination of techniques.

In this example, screening of useful stromal cell markers was done by immunocytochemistry of hES cells cultured in XVIVO 10™ with bFGF, or medium conditioned using mouse embryonic fibroblasts. Antibodies were obtained from commercial sources as follows:

TABLE 7: Primary Antibody for Measuring Differentiated Cells

Marker	Vendor	Catalog No.
STRO-1	RnD Systems	MAB 1038
Human Thymus Stroma	BD Pharmingen	555825
CD44	BD Pharmingen	550988
CD90	BD Pharmingen	555593
CD105 (Endoglin)	Chemicon	MAB2152
CD106 (VCAM-1)	BD Pharmingen	555645
Vimentin	Sigma	V 5255

Figure 8 shows the results of the immunocytochemical analysis. CD44, STRO-1 and Vimentin stain stromal-like cells in the hES cell populations cultured with mEF conditioned medium.

Example 8: Sensitivity of the assay for undifferentiated cells

Real-time PCR assays were performed using mixtures of undifferentiated hES cells and BJ fibroblasts, to determine the sensitivity of the assay for the presence of differentiated cells.

Freshly harvested cells were combined to a total of 2×10^6 cells in 10% increments of each cell type. Total RNA was isolated (Roche isolation kit), and then treated with DNAse 1 to remove potential DNA contaminants. (Ambion kit). Amplification mixtures were made up in QRT-PCR master mix buffer (P/N 4309169) to a final volume of 25 µL at a concentration of 10 µM forward primer, 10 µM reverse primer, 10 µM probe, and ~100 ng RNA. Data analysis was performed using the comparative Ct method using 18S rRNA endogenous control. (Other suitable housekeeping genes for standardization can be used instead, such as acidic ribosomal protein, β-actin, cyclophilin, G3P dehydrogenase, or β2-microglobulin).

Figure 9 shows the relative change of gene expression measured in mixtures of differentiated (BJ) and undifferentiated hES cells, compared with undifferentiated hES cells alone. These five markers are able to rank 10% changes in the proportion of undifferentiated cells.

SEQUENCE DATA

TABLE 8: Sequences Referred To in this Disclosure

Designation	Reference
hTERT mRNA sequence	GenBank Accession NM_003129
hTERT protein sequence	GenBank Accession NM_003129
Oct 3/4 mRNA sequence	GenBank Accession NM_002701
Oct 3/4 protein sequence	GenBank Accession NM_002701
Cripto mRNA sequence	GenBank Accession NM_003212
Cripto protein sequence	GenBank Accession NM_003212
podocalyxin-like protein mRNA sequence	GenBank Accession NM_005397
podocalyxin-like protein amino acid sequence	GenBank Accession NM_005397
GRP receptor mRNA sequence	GenBank Accession NM_005314
GRP receptor proteins sequence	GenBank Accession NM_005314
Primers & probes for real-time PCR assay	This disclosure
Human telomeric repeats	U.S. Patent 5,583,016
Novel expressed sequences from hES cells	This disclosure:SEQ. ID NOs:1-39

* * * * * * * * * * *

The subject matter provided in this disclosure can be modified as a matter of routine optimization, without departing from the spirit of the invention, or the scope of the appended claims.

CLAIMS

1. A method for assessing a culture of undifferentiated primate pluripotent stem (pPS) cells or their progeny, comprising detecting or measuring expression of three or more of the markers in any of Tables 5 to 9.

2. The method of the preceding claim, comprising measuring expression of three or more of the markers in Tables 2, 7, and 9(C), and correlating the expression measured with the presence of undifferentiated embryonic stem (ES) cells in the culture.

3. The method of any preceding claim, comprising measuring expression of three or more of the markers in Tables 3 and 8, and correlating the expression measured with the presence of differentiated cells in the culture.

4. The method of any preceding claim, comprising detecting or measuring expression of one or more of the following markers: bone marrow stromal antigen; Podocalyxin-like; Rat GPC/ glypican-2 (cerebroglycan); Potassium channel subfamily k member 5 (TASK-2); Notch··1 protein; Teratocarcinoma-derived growth factor 1 (Cripto); Nel 1 like / NELL2 (Nel-like protein 2); Gastrin releasing peptide receptor; Bone morphogenetic protein; ABCG2- ABC transporter; Solute carrier family 6, member 8 (SLC6A8); hTERT; Oct 3/4 Octamer-binding transcription factor 3a (Oct-3a) (Oct-4); Left-right determination factor b (LEFT); Secreted phosphoprotein 1 (osteopontin); Gamma-aminobutyric acid (GABA) A receptor, beta 3; Roundabout, axon guidance receptor, homologue 1 (ROBO1),; Glucagon receptor; Leucine-rich ppr-motif hum 130 kDa hum130leu 130kd leu; Thy-1 co-transcribed; Solute carrier family 21; LY6H lymphocyte antigen 6 complex locus H; Plexin (PLXNB3); Armadillo repeat protein deleted in velo-cardio-facial syndrome; and Ephrin type-a receptor 1 (EPHA1).

5. The method of any preceding claim, comprising detecting or measuring expression of three or more of said markers.

6. The method of any preceding claim, further comprising detecting or measuring expression of hTERT and/or Oct 3/4.

7. A method for assessing a culture of undifferentiated human embryonic stem (hES) cells or their progeny, comprising detecting or measuring two or more markers selected from Cripto, gastrin-releasing peptide (GRP) receptor, podocalyxin-like protein (PODXL), and human telomerase reverse transcriptase (hTERT).

8. The method of claim 7, further comprising detecting or measuring one or more markers selected from Oct 3/4, SSEA-3, SSEA-4, Tra-1-60 and Tra-1-81.

9. The method of claim 7 or 8, comprising detecting or measuring Cripto, hTERT, and Oct 3/4.

10. A method for assessing a culture of undifferentiated human embryonic stem (hES) cells or their progeny, comprising detecting or measuring two or more markers preferentially expressed in undifferentiated hES cells, and one or more markers expressed preferentially after differentiation of the hES cells.

11. The method of claim 10, wherein at least one of the markers preferentially expressed in undifferentiated hES cells is selected from Cripto, gastrin-releasing peptide (GRP) receptor, podocalyxin-like protein (PODXL), and human telomerase reverse transcriptase (hTERT).

12. The method of claim 10 or 11, wherein at least one of the markers preferentially expressed in undifferentiated hES cells is selected from Oct 3/4, SSEA-4, Tra-1-60 and Tra-1-81.

13. The method of claim 10 comprising detecting or measuring hTERT, Oct 3/4, and a marker selected from Cripto, SSEA-4, Tra-1-60 and Tra-1-81.

14. The method of any of claims 10-13, wherein at least one of the markers expressed preferentially after differentiation of the hES cells is a stromal cell markers.

15. The method of claim 14, wherein the stromal cell marker is selected from CD44, CD105 (endoglin), CD106 (VCAM-1), CD90 (Thy-1), STRO-1, Vimentin, and Human Thymus Stroma.

16. The method of any of claims 10-15, wherein expression of hTERT, Oct 3/4, Cripto, GRP receptor, PODXL, CD44, CD105, CD106, or CD90 is detected or measured at the mRNA level by PCR amplification.

17. The method of any of claims 10-16, wherein expression of SSEA-4, Tra-1-60, Tra-1-81, Cripto, Oct 3/4, CD44, CD105, CD106, CD90, STRO-1, Vimentin, or Human Thymus Stroma is detected or measured at the antigen expression level by antibody assay.

18. A kit for assessing a culture of pPS cells according to any of claims 1-6, comprising polynucleotide probes and/or primers for specifically amplifying a transcript for two or more markers in any of Tables 5 to 9, accompanied by written instructions for assessing the pPS cells according to the expression of said markers measured using the probes or primers in the kit.

19. A kit for assessing a culture of pPS cells according to any of claims 1-6, comprising an antibody specific for the gene product of two or more markers in any of Tables 5 to 9, accompanied by written instructions for assessing the pPS cells according to the expression of said markers measured using the antibody in the kit.

20. A kit for assessing a culture of undifferentiated human embryonic stem (hES) cells or their progeny according to claim 10, comprising antibody or PCR amplification primers specific for three or more markers, of which at least two are expressed preferentially in undifferentiated hES cells, and at least one is expressed preferentially in stromal cells.

21. The kit of claim 20, comprising antibody or PCR amplification primers specific for at least two markers selected from Cripto, gastrin-releasing peptide (GRP) receptor, podocalyxin-like protein (PODXL), human telomerase reverse transcriptase (hTERT) Oct 3/4, SSEA-4, Tra-1-60, Tra-1-81, CD44, CD105 (endoglin), CD106 (VCAM-1), CD90 (Thy-1), STRO-1, Vimentin, and Human Thymus Stroma.

22. Use of antibody or PCR amplification primers specific for three or more markers selected from Cripto, gastrin-releasing peptide (GRP) receptor, podocalyxin-like protein (PODXL), human telomerase reverse transcriptase (hTERT) Oct 3/4, SSEA-4, Tra-1-60, Tra-1-81, CD44, CD105 (endoglin), CD106 (VCAM-1), CD90 (Thy-1), STRO-1, Vimentin, and Human Thymus Stroma, for assessing a culture of undifferentiated human embryonic stem (hES) cells or their progeny.

23. The method of any of claims 1-17, which is a method for quantifying the proportion of undifferentiated pPS cells or differentiated cells in the culture.

24. The method of any of claims 1-17, which is a method for assessing the ability of a culture system or component thereof to maintain pPS cells in an undifferentiated state.

25. The method of claim 24, which is a method of assessing the ability of a soluble factor to maintain pPS cells in an undifferentiated state.

26. The method of claim 24, which is a method of assessing the ability of a culture medium to maintain pPS cells in an undifferentiated state.

27. The method of claim 24, which is a method of assessing the ability of a preparation of feeder cells to maintain pPS cells in an undifferentiated state.

28. The method of any of claims 1-17, which is a method for assessing the ability of a culture system or component thereof to cause differentiation of pPS cells into a culture of lineage-restricted precursor cells and/or terminally differentiated cells.

29. The method of any of claims 1-17, which is a method for assessing the suitability of a pPS cell culture for preparing cells for human administration.

30. The method of any of claims 1-17, wherein the level of the markers of undifferentiated hES cell markers is determined to be at least 100-fold higher than in BJ fibroblasts.

31. A method for assessing the growth characteristics of a cell population, comprising detecting or measuring expression of three or more of the markers in any of Tables 5 to 9.

32. A method for assessing the growth characteristics of a cell population, comprising detecting or measuring two or more markers selected from Cripto, gastrin-releasing peptide (GRP) receptor, podocalyxin-like protein (PODXL), and human telomerase reverse transcriptase (hTERT).

33. The method of claim 31 or 32, wherein the cell population has been obtained by culturing cells from a human blastocyst.

34. The method of claim 33, which is a method for determining whether the cell population is pluripotent.

35. The method of claim 31 or 32, wherein the cell population has been obtained from a human patient suspected of having a clinical condition related to abnormal cell growth.

36. The method of claim 31 or 32, which is a method for assessing whether the patient has a malignancy.

37. A method for maintaining pPS cells in a pluripotent state, comprising causing them to express one of the following markers at a higher level.
    - Forkhead box O1A (FOXO1A); Zic family member 3 (ZIC3); Hypothetical protein FLJ20582; Forkhead box H1 (FOXH1); Zinc finger protein, Hsal2; KRAB-zinc finger protein SZF1-1; and Zinc finger protein of cerebellum ZIC2.

38. The method of claim 37, wherein the cells are caused to express the marker by genetically altering it with a gene that encodes the marker.

39. A method for causing pPS cells to differentiate into a particular tissue type, comprising causing them to express one of the following markers at an altered level.
    - Forkhead box O1A (FOXO1A); Zic family member 3 (ZIC3); Hypothetical protein FLJ20582; Forkhead box H1 (FOXH1); Zinc finger protein, Hsal2; KRAB-zinc finger protein SZF1-1; Zinc finger protein of cerebellum ZIC2; and Coup transcription factor 2 (COUP-TF2).

40. The method of claim 39, wherein the cells are caused to express the marker by genetically altering it with a gene that encodes the marker, or with an antisense nucleic acid that binds to mRNA encoding the marker.

41. A method for causing an encoding sequence to be preferentially expressed in undifferentiated pPS cells, comprising genetically altering pPS cells with the encoding sequence under control of a promoter for one of the markers listed in any of Tables 2, 7, and 9(C).

42. The method of claim 41, which is a method for selecting undifferentiated cells, and the encoding sequence is a reporter gene (such as a gene that causes the cells to emit fluorescence), or a positive selection marker (such as a drug resistance gene).

43. The method of claim 41, which is a method for depleting undifferentiated cells from a population of differentiated cells, and the encoding sequence is a negative selection marker (such as a gene that activates apoptosis or converts a prodrug into a compound that is lethal to the cell).

44. A method for causing an encoding sequence to be preferentially expressed in differentiated cells, comprising genetically altering the pPS cells with the encoding sequence under control of a promoter for one of the markers listed in Table 3 or Table 8.

45. The method of claim 44, which is a method for selecting differentiated cells, and the encoding sequence is a reporter gene (such as a gene that causes the cells to emit fluorescence), or a positive selection marker (such as a drug resistance gene).

46. The method of claim 44, which is a method for depleting differentiated cells from a population of undifferentiated cells, and the encoding sequence is a negative selection marker (such as a gene that activates apoptosis or converts a prodrug into a compound that is lethal to the cell).

47. A method for sorting differentiated cells from less differentiated cells, comprising separating cells expressing a surface marker in any of Tables 5 to 9 from cells not expressing the marker.

48. The method of claim 47, wherein the cells are sorted using an antibody or lectin that binds the marker or product thereof on the cell surface.

49. A method for causing pPS cells to proliferate without differentiation, comprising culturing them in a culture system assessed according to the method of claim 7.

50. A method for causing pPS cells to proliferate without differentiation, comprising culturing them with human mesenchymal stem cells.

51. A method for identifying genes that are up- or down-regulated during differentiation of pPS cells, comprising:

a) sequencing transcripts in an expression library from undifferentiated pPS cells;

b) sequencing transcripts in one or more expression libraries from one or more cell types that have differentiated from the same line of pPS cells;

c) determining the frequency of transcripts from each gene sequenced in each of the libraries;

d) identifying the gene as being up- or down-regulated during differentiation of the pPS cells if the frequency of transcripts in the library from the undifferentiated pPS cells is different from the frequency of transcripts in one or more libraries from the differentiated cell types at a statistical probability of at least 95%.

52. The method of claim 51, further comprising assessing a culture of pPS cells depending on the expression level measured in cells from the culture of the marker identified in d).

53. The method or use according to any of claims 1-17 or 22-54, wherein the pPS cells are obtained from a human blastocyst, or are the progeny of such cells.

54. The method or use of claim 53, wherein the pPS cells are human embryonic stem cells.

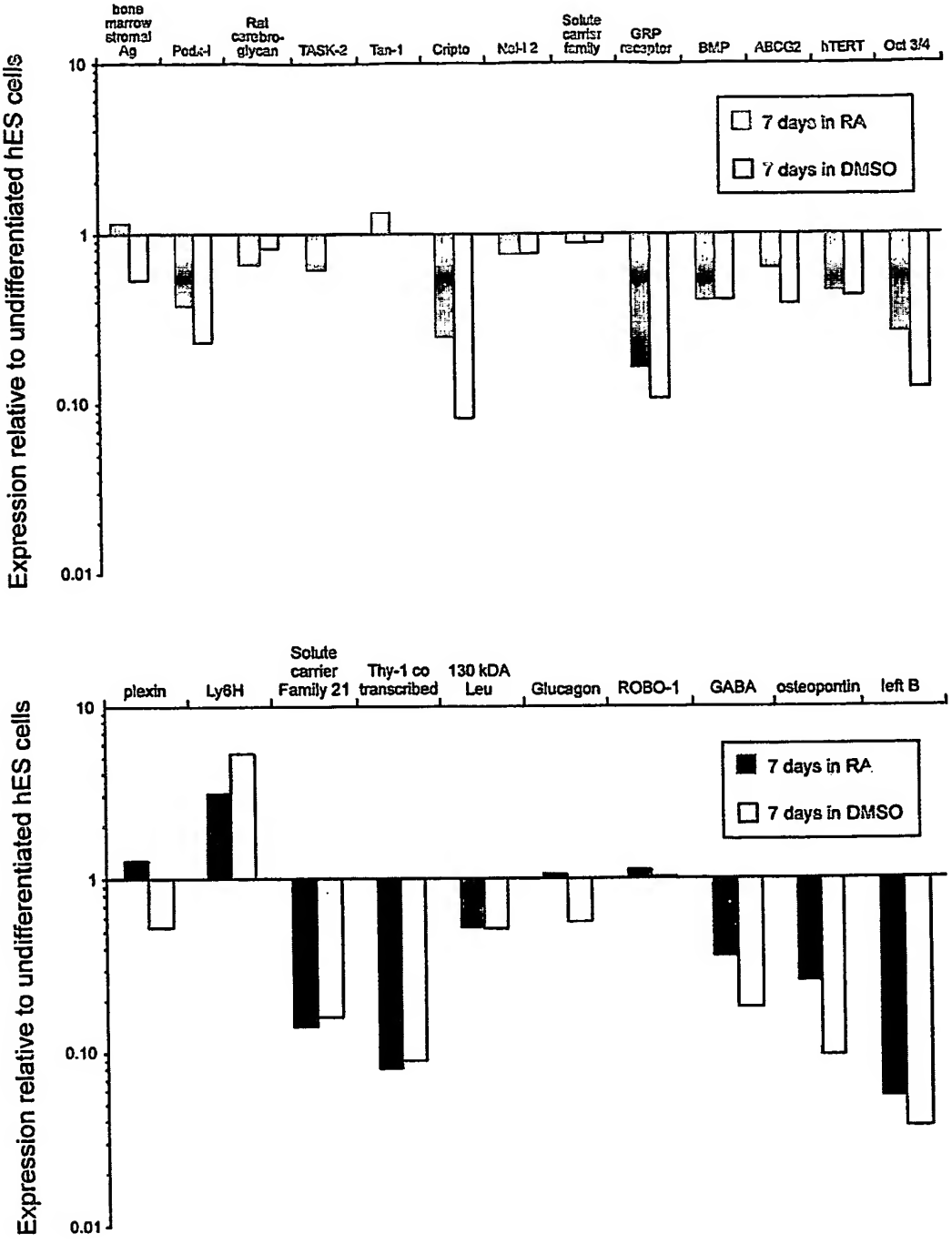
55. The kit of according to any of claims 18-21, wherein the pPS cells are obtained from a human blastocyst, or are the progeny of such cells.

56. The kit of claim 55, wherein the pPS cells are human embryonic stem cells.

57. The method or use according to any of claims 1-17 or 22-54, substantially as hereintofore described with reference to any one of the Examples.

58. The kit according to any of claims 18-21, substantially as hereintofore described with reference to any one of the Examples.

Figure 1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Podx-l
Rat cerebro-glycan
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Cripto
Nch-l 2
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GRP receptor
BMP
ABCG2
hTERT
Oct 3/4
10
1
0.10
0.01
Expression relative to undifferentiated hES cells
7 days in RA
7 days in DMSO
plexin
Ly6H
Solute carrier Family 21
Thy-1 co transcribed
130 kDA Leu
Glucagon
ROBO-1
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10
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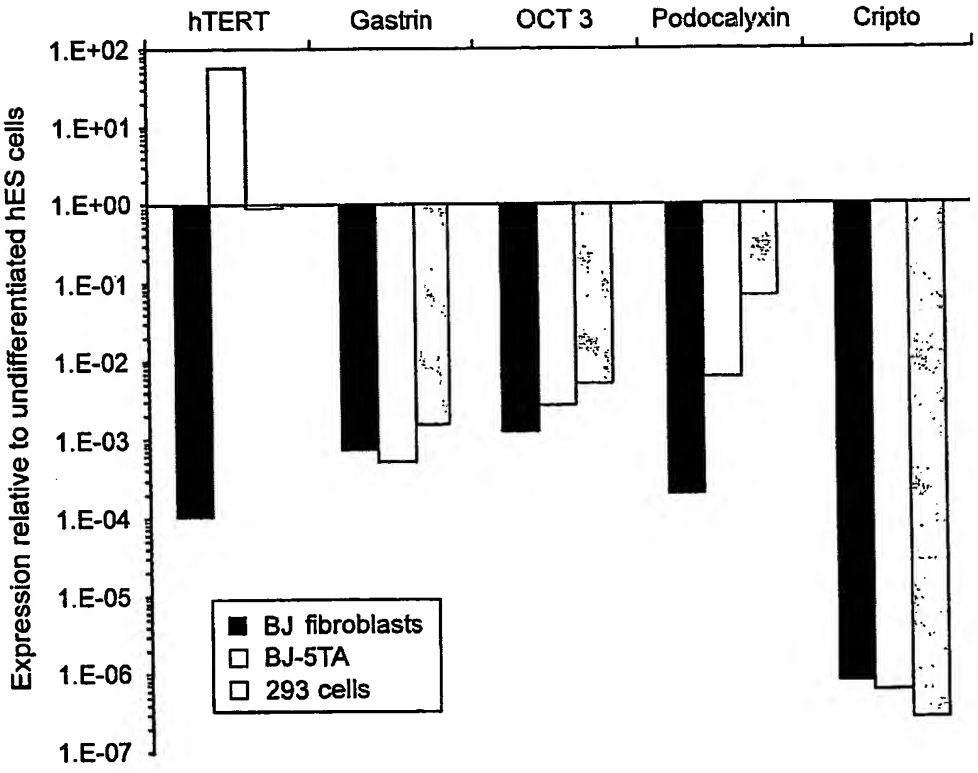
Expression relative to undifferentiated hES cells
7 days in RA
7 days in DMSO

Figure 2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Gastrin
OCT 3
Podocalyxin
Cripto
1.E+02
1.E+01
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1.E-01
1.E-02
1.E-03
1.E-04
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1.E-07
Expression relative to undifferentiated hES cells
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BJ-5TA
293 cells

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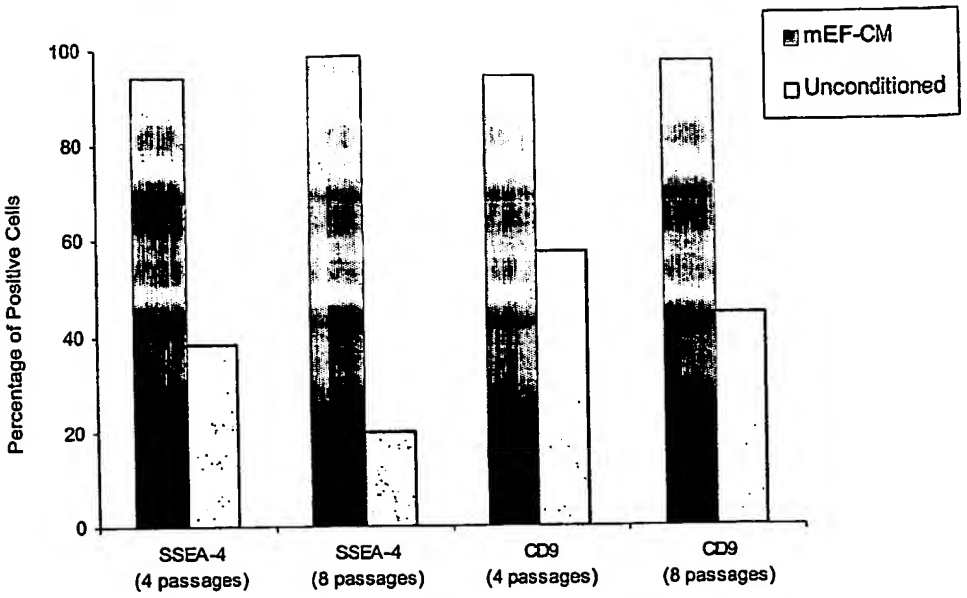

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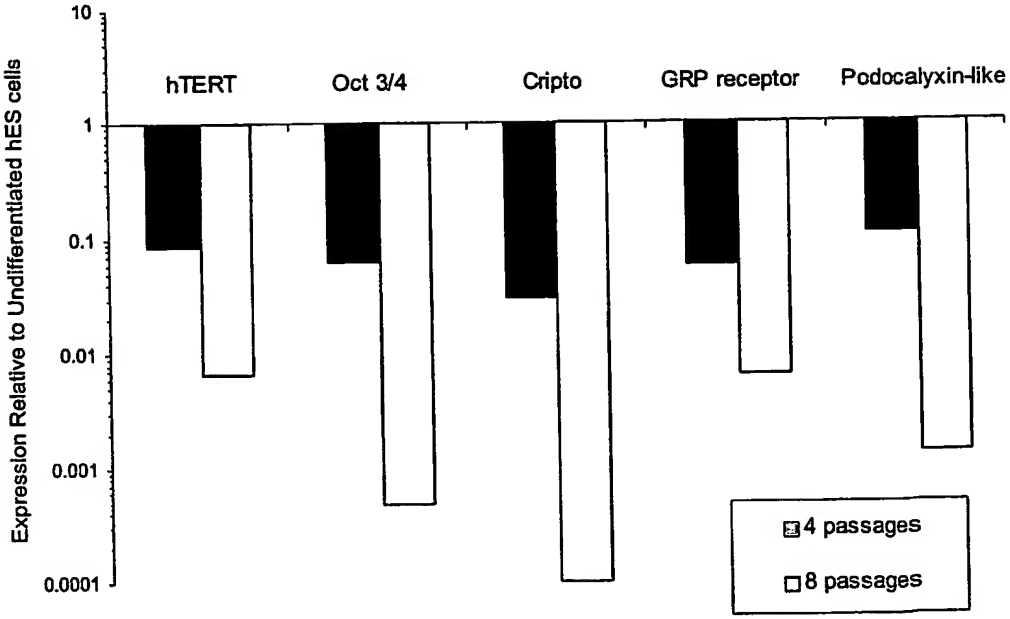

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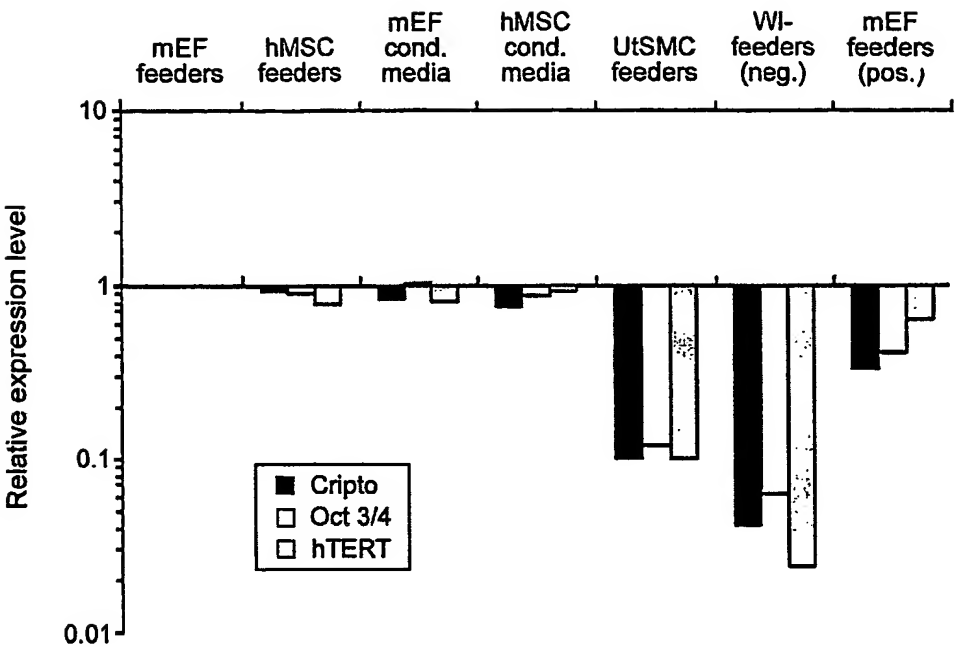
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mEF cond. media
hMSC cond. media
UtSMC feeders
WI-feeders (neg.)
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Relative expression level
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0.1
0.01
Cripto
Oct 3/4
hTERT

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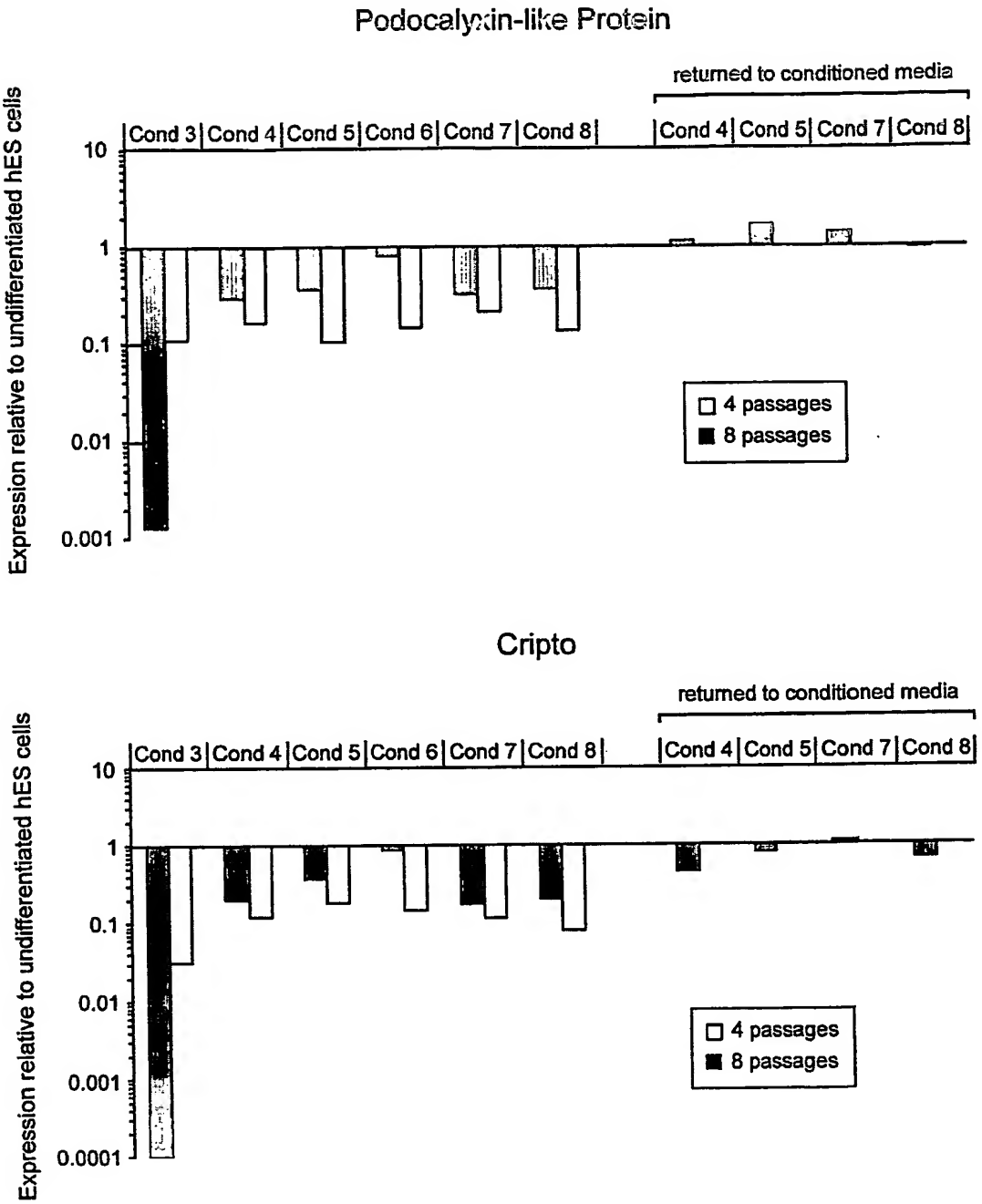
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Cond 4
Cond 5
Cond 6
Cond 7
Cond 8
Cond 4
Cond 5
Cond 7
Cond 8
Expression relative to undifferentiated hES cells
10
1
0.1
0.01
0.001
4 passages
8 passages
Cripto
returned to conditioned media
Cond 3
Cond 4
Cond 5
Cond 6
Cond 7
Cond 8
Cond 4
Cond 5
Cond 7
Cond 8
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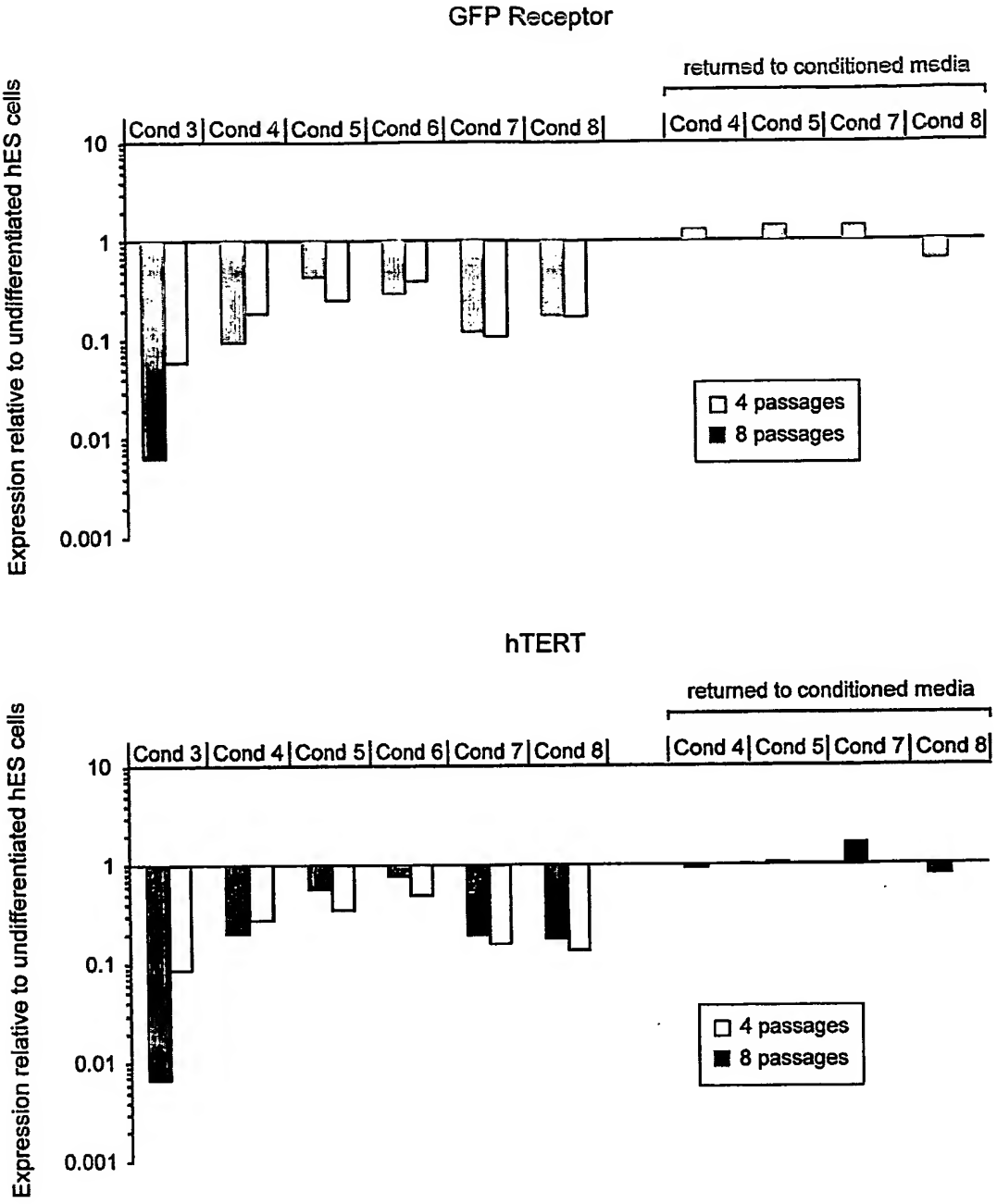
10
1
0.1
0.01
0.001
0.0001
4 passages
8 passages

Figure 6(B)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Cond 4
Cond 5
Cond 6
Cond 7
Cond 8
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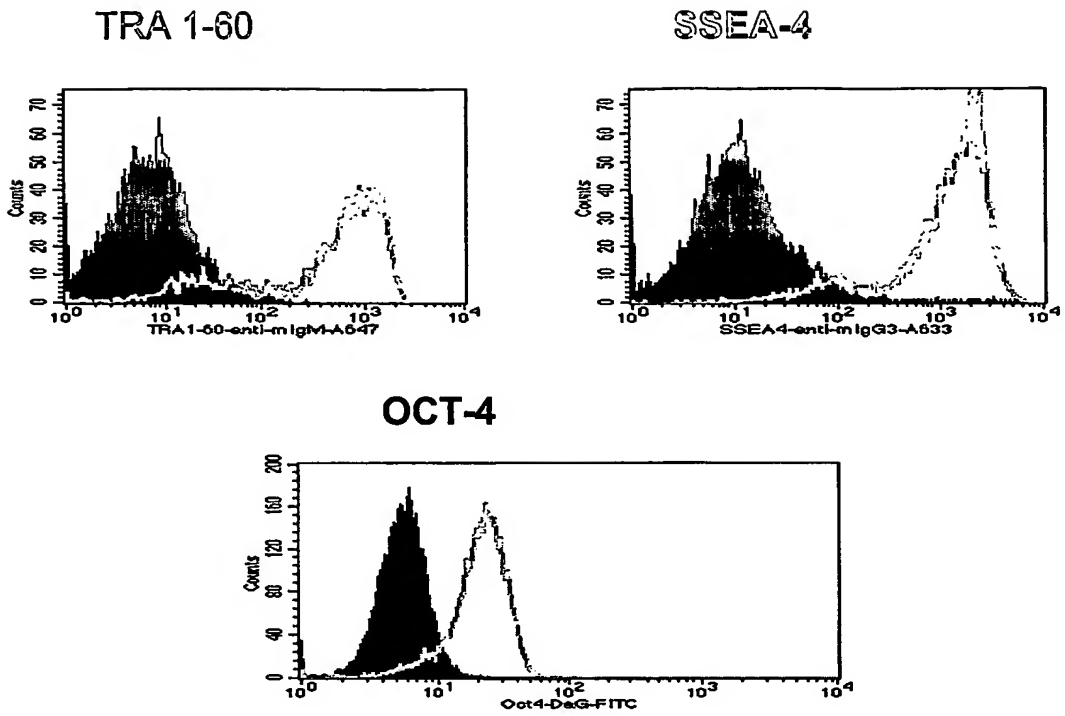
Cond 7
Cond 8
Expression relative to undifferentiated hES cells
10
1
0.1
0.01
0.001
4 passages
8 passages
hTERT
returned to conditioned media
Cond 3
Cond 4
Cond 5
Cond 6
Cond 7
Cond 8
Cond 4
Cond 5
Cond 7
Cond 8
Expression relative to undifferentiated hES cells
10
1
0.1
0.01
0.001
4 passages
8 passages

Figure 7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OCT-4
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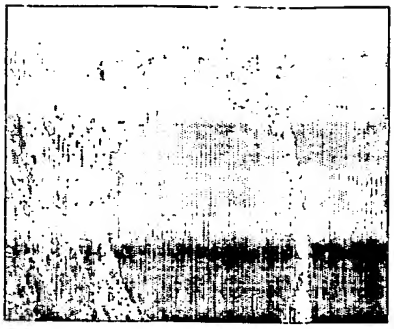
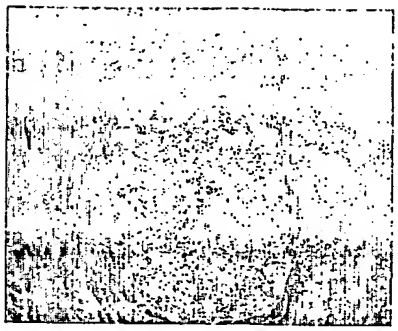
Figure 8

CD44





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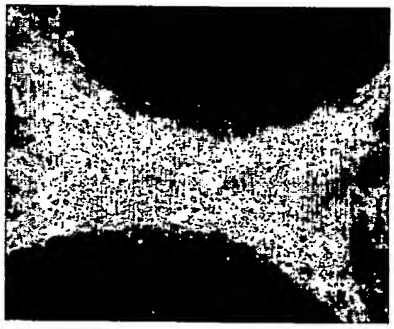
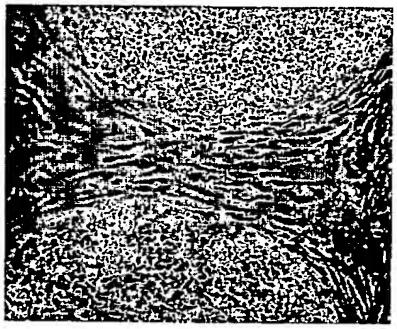
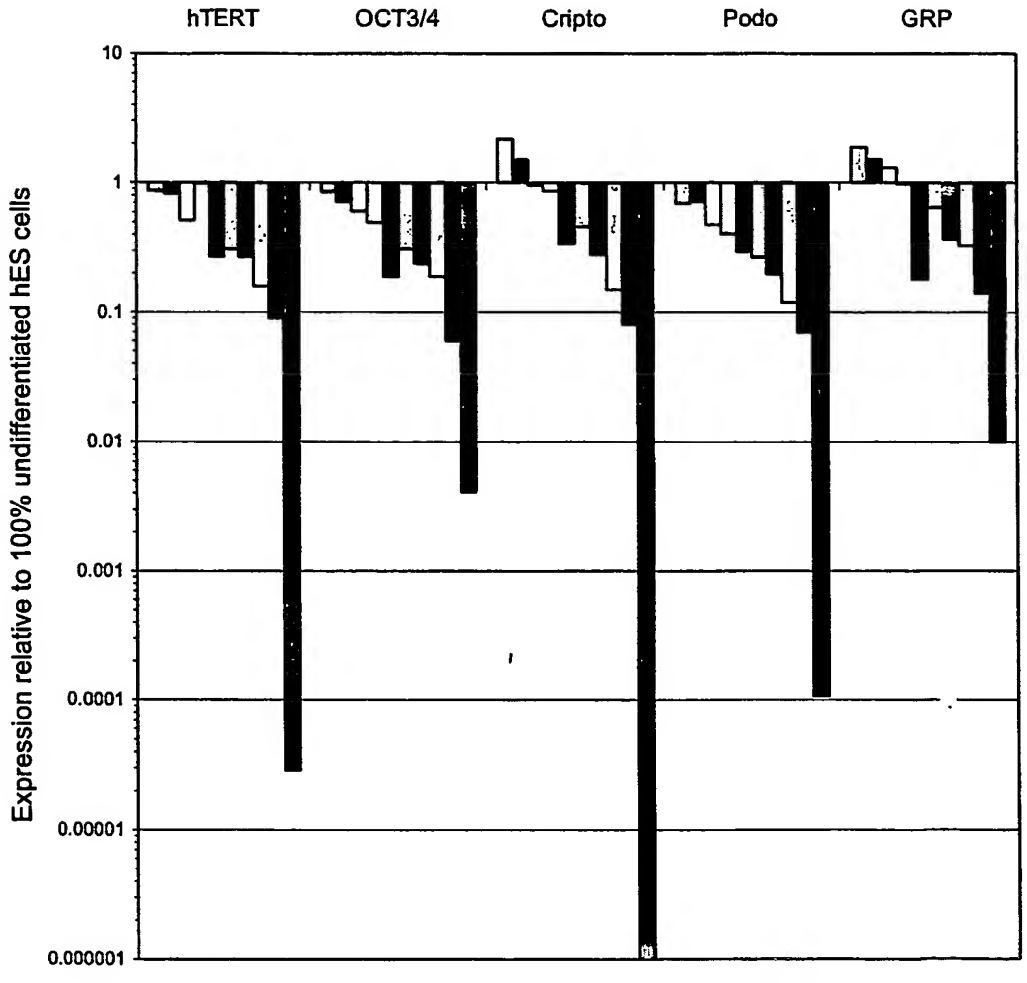




Vimentin

Figure 9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OCT3/4
Cripto
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135300 - SeqList

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<212> DNA
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(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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(54) Title: A MARKER SYSTEM FOR CHARACTERIZING UNDIFFERENTIATED HUMAN EMBRYONIC STEM CELLS

(57) Abstract: This disclosure provides a system for qualifying embryonic stem cells intended for human therapy. A large-scale sequencing project has identified important markers that are characteristic of undifferentiated pluripotent cells. Combinations of these markers can be used to validate the self-renewing capacity of ES cells, and their ability to differentiate into tissue types suitable for regenerative medicine. The marker system of this invention has been used to screen feeder cells, media additives, and culture conditions that promote proliferation of stem cells without differentiation. A culture system optimized by following these markers is suitable for rapid expansion of undifferentiated cells from existing lines, or the derivation of new lines that are equally apposite for clinical use.

WO 2004/080146 A3

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International Application No
PCT/EP2004/002808

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/53

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	US 2003/224411 A1 (STANTON LAWRENCE W ET AL) 4 December 2003 (2003-12-04) claim 1	1,3-5, 23,53, 54,57
Y	WO 00/27995 A (PERA MARTIN FREDERICK ; REUBINOFF BENJAMIN EITHAN (AU); TROUNSON ALAN) 18 May 2000 (2000-05-18) claims 6,7	1
Y	WO 02/102837 A (KERJASCHKI DONTSCHO ; INNOVATIONSAGENTUR GES M B H (AT); KEROSUO LAURA) 27 December 2002 (2002-12-27) claims 14,15	1

-/—

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 April 2005	22 07. 2005

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Hinchliffe, P

Form PCT/ISA/210 (second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2004/002808

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHENG LINZHAO ET AL: "Human adult marrow cells support prolonged expansion of human embryonic stem cells in culture." STEM CELLS (MIAMISBURG), vol. 21, no. 2, 2003, pages 131-142, XP002323378 ISSN: 1066-5099 see Table 1	10-14
X	THOMSON J A ET AL: "EMBRYONIC STEM CELL LINES DERIVED FROM HUMAN BLASTOCYSTS" SCIENCE, AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE,, US, vol. 282, 6 November 1998 (1998-11-06), pages 1145-1147, XP002121340 ISSN: 0036-8075 page 1146, column 1, line 1 - line 10	10-14
X	XU CHUNHUI ET AL: "Feeder-free growth of undifferentiated human embryonic stem cells" NATURE BIOTECHNOLOGY, NATURE PUBLISHING, US, vol. 19, no. 10, October 2001 (2001-10), pages 971-974, XP002282070 ISSN: 1087-0156 cited in the application page 972, column 1, line 16 - page 972, column 2, line 53	10-14
X	DEANS ROBERT J ET AL: "Mesenchymal stem cells: Biology and potential clinical uses" EXPERIMENTAL HEMATOLOGY, NEW YORK, NY, US, vol. 28, no. 8, August 2000 (2000-08), pages 875-884, XP002201188 ISSN: 0301-472X see table 1	10-14
A	RICHARDS M ET AL: "Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells" NATURE BIOTECHNOLOGY, NATURE PUBLISHING, US, vol. 20, no. 9, 5 August 2002 (2002-08-05), pages 933-936, XP002979805 ISSN: 1087-0156 figure 2	10-14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2004/002808

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [X] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

10-14

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[X] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.1

Although claims 31-36 are directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Although claims 37-46 can also be directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Table 9, referred to in the claims, does not appear in the descripition. Consequently the claims are unclear contrary to Article 5 PCT insofar as they relate to non existent table 9.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1,3,4,5,23,53,54,57 (all partly)

   A method for assesing (if) a culture comprises undifferentiated pluripotent stem cells by measuring three or more markers selected from tables 5-8 (table 9 could not be found).
   The first three markers in table 5 are bone marrow stromal antigen, podocalyxin and Rat GPC/glypian-2.

---

2. claims: 1,3,4,5 (partly)

   A method for assesing (if) a culture comprises undifferentiated pluripotent stem cells by measuring three or more markers selected from tables 5-8 (table 9 could not be found).
   Bearing in mind that tables 5-8 include 49 different markers, then the sum of each triplet must be individually searched.

---

3. claim: 2 (partly)

   A method for assessing (if) a culture comprises undifferentiated pluripotent stem cells by measuring three or more markers selected from tables 5-8 (table 9 could not be found) and table 2.
   Bearing in mind that tables 5-8 include 49 different markers and table 2 an additional 30 markers, then the sum of each triplet must be individually searched.

---

4. claim: 6 (partly)

   A method for assessing (if) a culture comprises undifferentiated pluripotent stem cells by measuring three or more markers selected from tables 5-8 (table 9 could not be found) and table 2.
   Bearing in mind that tables 5-8 include 49 different markers and table 2 an additional 30 markers, then the sum of each triplet must be individually searched. In addition these combinations must be further combined with either hTERT and/or Oct 3/4. The number of possibilities to search is now an enormous task.

---

5. claim: 7 (partly)

   A method for assessing (if) a culture comprises undifferentiated pluripotent stem cells by measuring two or more markers selected from Cripto, GRP, PODPXL, hTERT. Seven (7) combinations must be searched

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

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6. claim: claim 8 (partly)

A method for assessing (if) a culture comprises undifferentiated pluripotent stem cells by measuring two or more markers selected from Cripto, GRP, PODPXL, hTERT, Oct3/4, SSEA-3, SSEA-4, Tra-1-60 and Tra-1-81. Again the sum of the combinations to be searched is very large.

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7. claim: 9

A method for assessing (if) a culture comprises undifferentiated pluripotent stem cells by measuring three markers : Cripto, hTERT and Oct3/4.

---

8. claims: 10, 47,48 (partly)

A method for assessing (if) a culture comprises undifferentiated pluripotent stem cells by measuring two or more markers preferentially expressed by said cells and IN ADDITION measuring one or more marker(s) preferentially expressed in differentiated stem cells.
Again all of the markers given in tables 5 -8 (no 9) must be individually searched.

---

9. claim: 11

A method for assessing (if) a culture comprises undifferentiated pluripotent stem cells by measuring two or more markers preferentially expressed by said cells and IN ADDITION measuring one or more marker(s) preferentially expressed in differentiated stem cells. Undifferentiated markers are chosen form Cripto, GRP, PODXL, hTERt

---

10. claim: 12

A method for assessing (if) a culture comprises undifferentiated pluripotent stem cells by measuring two or more markers preferentially expressed by said cells and IN ADDITION measuring one or more marker(s) preferentially expressed in differentiated stem cells. Markers include Oct 3/4, SSEA-4, Tra-1-60, Tra-1-81.

---

11. claim: 13

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

A method for assessing (if) a culture comprises undifferentiated pluripotent stem cells by measuring two or more markers preferentially expressed by said cells and IN ADDITION measuring one or more marker(s) preferentially expressed in differentiated stem cells. Markers include Oct 3/4, SSEA-4, Tra-1-60, Tra-1-81, Cripto, hTERT.

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12. claim: 14 (partly)

A method for assessing (if) a culture comprises undifferentiated pluripotent stem cells by measuring two or more markers preferentially expressed by said cells and IN ADDITION measuring one or more marker(s) preferentially expressed in differentiated stem cells. Markers include Oct 3/4, SSEA-4, Tra-1-60, Tra-1-81.
In addition a stomal marker is used for the differentiation marker.

---

13. claim: 15 (partly)

As claim 14 but the stromal markers are selected from the group comrising: CD44, CD105,CD106, CD90, STRO-1, Vimentin, HTS.

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14. claim: 16 (partly)

sub selection of lists in previous claims 10-15

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15. claims: 17,22,55,56,58 (all partly)

sub selection of lists in previous claims 10-16 with a use of antibodies or primers to said markers being included.

---

16. claim: 18 (partly)

Kits comprising two or more probes/primers selected from the markers given in tables 5-8 (no 9).
The kits are not characterised by their instructions and therefore are merly primer/probe pairs for any use.

---

17. claim: 19 (partly)

Kits comprising two or more antibodies reactive with the markers given in tables 5-8 (no 9).
The kits are not characterised by their instructions and therefore are merly antibody pairs for any use.

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18. claims: 20,21 (partly)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Kits comprising two or more antibodies/PCR primers selected from the markers given in tables 5-8 (no 9).
The kits are not characterised by their instructions and therefore are merly antibody/primer pairs for any use.

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19. claims: 24,25,26,27,49

A method for testing to see which substances/cells/culture conditions are able to keep hES in an undifferentiated state. A method of keeping cells in said undifferentiated form using said substances.

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20. claim: 28

A method for testing to see which substances/cells/culture conditions are able to differentiate hES in a specific manner.

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21. claim: 29

A method for testing to see which substances/cells/culture conditions are suitable for human admininstration.

---

22. claim: 30

Comparison of the levlel of all of the markers listed with those in BJ fibroblast cells.

---

23. claims: 31 (partly), 32,33,34,35,36

A method for assessing the growth characteristics of a cell population by looking for three or more of the markers given in tables 5 to 8 (no 9) or a specific selection from said tables.

---

24. claims: 37,38

A method of maintaining pluripotent stem cells in said state by causing them to express FOXO1A or ZIC3 or FLJ20582 or FOXH1 or (any) zink finger protein or Hsa1 or KRAB or SZF1-1 and ZIC2.

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25. claims: 39,40

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

A method of causing pluripotent stem cells to differentiate by causing them to alter their expresion lelvels of FOXO1A or ZIC3 or FLJ20582 or FOXH1 or (any) zink finger protein or Hsa1 or KRAB or SZF1-1 and ZIC2.

---

26. claims: 41,42,43 (all partly)

A method of using the promotoers associated with any of the markers given in tables 5-8 (no 9) to be used to express a heterologous gene in pPS cells.

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27. claims: 44-46 (all partly)

A method of using the promoters associated with any of the markers given in tables 3 and 8 to be used to express a heterologous gene in pPS cells.

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28. claim: 50

A method of causing pPS cells to grow without differentiation by co culture with mesenchymal stem cells.

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29. claims: 51,52

A method of identifiyng genes differentially regulated in pPS cells that have undergone differentiation using routine expression level comparisons.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP2004/002808

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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